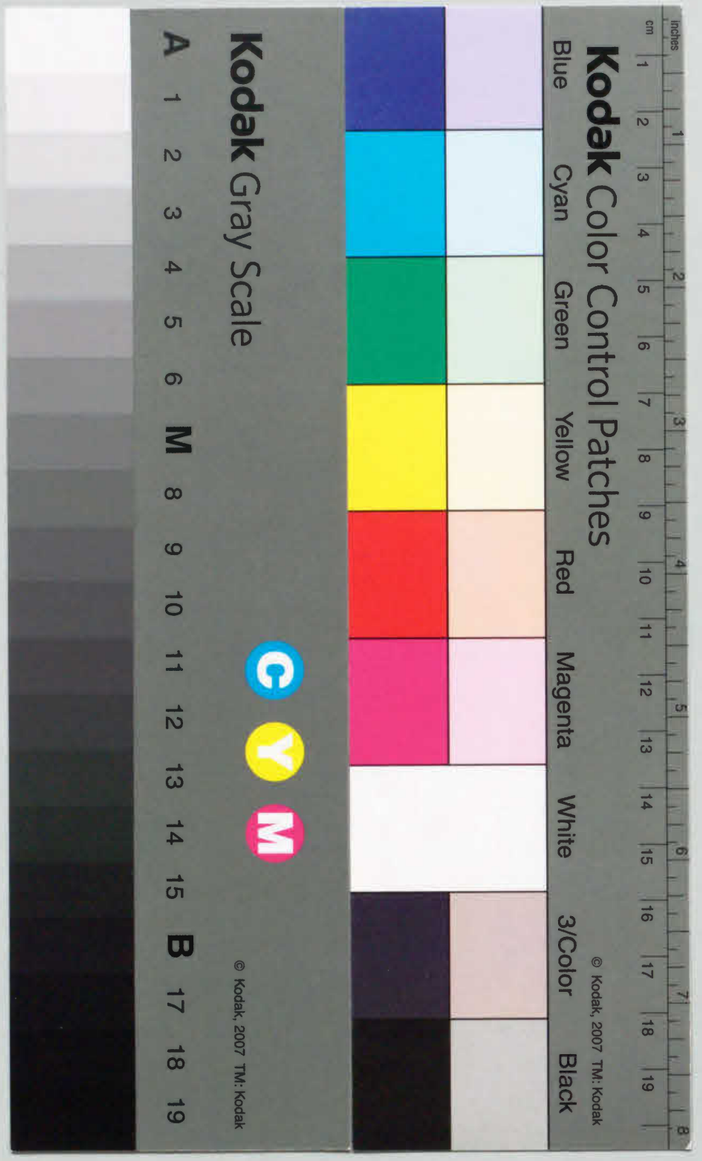

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学位論文題目	Studies on antimicrobial quaternary ammonium compounds		

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2

論 文 内 容 要 旨

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学位論文題目	Studies on antimicrobial quaternary ammonium compounds		

内容要旨

この論文は抗微生物活性を有する第四アンモニウム塩（QAC）の合成とその活性特性に関する研究報告である。この研究目的は、高い抗微生物活性と広い活性スペクトルを有する優れた薬剤の化学構造をデザインし、さらにQACの殺菌機構解明の糸口を得ることである。本研究では多種多様な化学構造を有するQACの中で、塩化ベンザルコニウムに代表される脂肪族QACとは異なり、合成が比較的容易で、殺菌機構解明が進みやすいと考えられるピリジニウム型のQACに着目した。

第１章では、*N*-置換アルキル基の炭素数を12に固定し、ピリジン核に様々な置換基を導入したQACを合成し、定量的構造活性相関の手法により優れた活性を有する薬剤を探索した。ピリジニウム型QACの殺菌活性は、置換基の種類と導入ポジションに大きく影響を受け、特にアミノ基やメチル基のような電子供与性基が2位、4位に導入されることによって高められることが明らかとなった。また殺菌特性は、各誘導体のピリジンの酸解離定数と、４級窒素に隣接するメチレンプロトンのケミカルシフト値と高い相関関係があった。これらの結果より、ピリジニウム型QACの殺菌活性が４級窒素の電子密度に依存することがわかった。

一方、*Escherichia coli* K12の細胞懸濁液は上記薬剤と接触すると短時間で濁りを生じた。この現象は薬剤が引き起こした細菌細胞膜の破壊が原因であると考えられた。そこでこの現象を引き起こす薬剤の作用を細胞破壊活性と定義し、殺菌活性に対してプロットすると高い相関関係が得られた。これより、この細胞破壊現象がQACの殺菌機構の重要な１段階であり、殺菌作用に大きく影響していることが示唆された。

2～4章では、1章で得られた知見をもとに、ピリジン核の2位または4位にベンジルアミノ基、アリルチオ基、あるいはアルキルチオ基を導入し、*N*-置換アルキル基の炭素数を変化させてそれらの抗微生物活性を測定した。各薬剤は置換基としてアルキル基しか持たない基本骨格の*N*-アルキルピリジニウム塩よりも高い殺菌活性と細胞破壊活性を示し、各QACの活性はアルキル鎖長、すなわち薬剤分子の疎水性に依存した。また、ベンジルアミノ基のような比較的大きな置換基の導入によって、広いpH領域で高い殺菌活性が維持された。さらに、アルキルチオ基の導入で1分子中に2個のアルキル基を持ったQACは、殺菌温度の影響をほとんど受けなかった。

5章は、2個のアンモニウム窒素と2個の*N*-アルキル基を有するbis-QACの合成およびその抗微生物活性の結果である。先の章で得られた知見から、かさだかい置換基を持つQACが優れた殺菌特性を持ったことより、分子の大きなbis-QACが高い抗微生物活性を有することが予期された。2つのアルキルピリジニウム塩を4位でイオウを介して3～10個のメチレン鎖で連結した化学構造を持つ新規bis-QACは、グラム陰性細菌、陽性細菌の両方に対して非常に高い殺菌活性を示した。また、一般にQACはカビに対して有効ではないが、新規bis-QACは代表的な抗カビ剤のTBZよりも高い活性を示した。さらにbis-QACはpH、温度の影響を受けず高い殺菌活性を維持した。

Studies on Antimicrobial Quaternary Ammonium Compounds

Kiyo Ohsaki

November 1968

Studies on Antimicrobial Quaternary Ammonium Compounds

Kiyo Okazaki

November 1999

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Introduction

Since Domagk (1935) reported the antibacterial activity of the quaternary ammonium compounds (QACs), the commercial importance of them as medicines, disinfectants, antimicrobials and many other applications was rapidly developed. Nowadays various types of the QACs are used widely in the food and textile industries, and in hospitals. They have several advantages over other types of biocides and disinfectants in that they possess higher antimicrobial activity, broader spectra of activity and lower toxicity.

QACs can be classified chemically by the number of quaternary nitrogen atoms in the molecule, that is, mono-QAC, bis-QAC and polymeric QAC. The synthesis and the antimicrobial characteristics of various series of mono-QACs have been reported by many researchers: Kourai et al. (1970; 1972; 1973; 1980b; 1983a; 1986b), Csiba et al. (1987), Kopecka-Leitmanova et al. (1989), Krysinski (1990; 1991), Devinsky et al. (1987; 1991) and Zamocka et al. (1994).

The activity of mono-QACs is generally influenced by structural features such as *N* substituted group and alkyl group. For instance, *N*-alkylbenzyldimethylammonium chloride (benzalkonium chloride), the most commercially successful product, shows the highest activity with an alkyl chain length of 14 in the homologous series (Daoud et al., 1983). The modification of benzalkonium chloride by substitution of aromatic ring hydrogen with chlorine, methyl, and ethyl groups gave an efficacious biocide, alkyldimethyl-3,4-dichlorobenzylammonium

chloride (Merianos, 1991). *N*-Alkylpyridinium derivatives were also affected by the kind of substituent groups and their positions on the pyridine ring (Kourai et al., 1980a). The relationships between physicochemical properties and the antimicrobial activity of mono-QACs against a variety of microorganisms were investigated. Daoud et al. (1983) reported the parabolic relationships between molecular hydrophobicity (log P) obtained from an octyl alcohol-water partition coefficient (P) and antimicrobial activity of benzalkonium chloride. Such parabolic relationships have been demonstrated by Hansch and Fujita (1964) and Hansch and Clayton (1973). Similarly quantitative structure-activity relationship (QSAR) analysis indicated that the antimicrobial activity of mono-QACs depends on the critical micelle concentration (Tomlinson et al., 1977; Devinsky et al., 1985) and the acidic dissociation constant (pKa) of the corresponding pyridine (Kourai et al., 1985b).

On the other hand, there is relatively little information about the antimicrobial characteristics of bis-QACs. The synthesis and the antimicrobial activity of bis-QACs have been reported by Devinsky et al. (1985; 1986) and Mlynarcik et al. (1981). In 1986, Kourai et al. (1986a) reported that *N,N'*-dialkyl-γ,γ'-dipyridinium diiodides (PP-n,n) exhibited stronger antibacterial activity than mono-QAC. In addition, the antimicrobial characteristics of PP-n,n, with two similar alkyl chains, were comparable to those of QACs with two different alkyl chains. Pavlikova-Moricka et al. (1994) found that the bis-QACs derived from bis-(2-dimethylaminoethyl) ester of glutaric acid

possessed high antimicrobial activity particularly to gram-negative bacteria. These reports indicate that bis-QACs can possess strong activity.

The synthesis and antimicrobial activity of polymeric QACs, as well as those of mono-QACs, have been investigated by many researchers (Ghosh, 1986; Rembaum, 1973; Gall et al., 1972; Kourai et al., 1994b). These polymeric QACs have linear or branched chemical structures and high molecular weight (MW), and electrostatically react to negatively charged surfaces of materials and organisms. The linear polymeric QACs with positively charged nitrogen atoms located in the backbone of polymeric chains have a much stronger antimicrobial activity than cross-linked QACs (Nakagawa et al., 1982; 1984a,b). Ikeda and Tazuke (1983; 1984; 1985) studied the effect of MW of synthesized polycations on antibacterial activity and found an optimal MW. They also reported that antimicrobial activity of poly[trialkyl(vinylbenzyl)ammonium chloride] against bacteria and fungi was higher than that of the corresponding mono-QACs, particularly against gram-positive bacteria. The antimicrobial activity of a series of soluble pyridinium-type polymers was investigated by Li et al. (1998). The polymers had only very weak toxicity (LD_{50} = 2,330 mg/kg).

However, the mode of action of QACs has not been completely elucidated. At the present stage of study, it may be summarized as follows (Ikeda and Tazuke, 1985): 1) Adsorption onto the bacterial cell surface, 2) Diffusion through the cell wall, 3) Binding to the

cytoplasmic membrane, 4) Disruption of the cytoplasmic membrane, 5) Release of K^+ ions and other cytoplasmic constituents, 6) Precipitation of cell contents and the death of the cells.

This thesis deals with the synthesis and antimicrobial characteristics of new QACs. The aim of this study is to obtain the information to design the most excellent QACs with potent and broad antimicrobial activity and spectra and high stability against various test conditions (time of contact with microbes, temperature and pH of the test medium, etc.). The final purpose is to understand the mode of action of QACs as antimicrobials.

The investigation of the pyridinium type of QACs has not yet been completely described in detail. Information obtained from the synthesized compounds must be abundant and significant to know the mode of the antimicrobial action of QACs. Therefore the synthesis of pyridinium compounds having various chemical structures were attemped and the antimicrobial characteristics of the synthesized compounds were investigated.

Chapter 1 describes the QSAR study of *N*-dodecylpyridinium iodide derivatives having various substituents (electron-attracting or electron-releasing groups) on the pyridine ring. Their activity was analyzed with their physicochemical parameters such as the acidic dissociation constant (pKa), the chemical shift (δ ppm) of ^{1}H-NMR and their molecular hydrophobicity (R_M). Further the turbidity phenomenon of bacterial cell suspension added the QACs, which closely relate to the bactericidal mechanism, was also investigated.

Chapters 2-4 concern the effects of the substituent groups on the pyridine ring on the antibacterial characteristics of mono-QACs. *N*-Alkyl-2-benzylaminopyridinium iodides having an electron-releasing group, *N*-alkyl-4-allylthiopyridinium bromide having an unsaturated substituent, and *N*-alkyl-2-alkylthiopyridinium and *N*-alkyl-4-alkylthiopyridinium salts having two long alkyl chains in the molecule were synthesized, respectively. These compounds could be expected to possess high antimicrobial activity. Their activity was measured and compared with *N*-alkylpyridinium iodides having only alkyl group as a substituent on the pyridine ring.

Chapter 5 describes the synthesis and the antimicrobial activity of new bis-QAC, 4,4'-(α, ω-polymethylenedithio)bis(1-alkylpyridinium iodide)s, having two active moieties for bactericidal action and two long alkyl chains in the molecule. Their bactericidal activity was very high and the bactericidal properties were different from those of mono-QACs.

Chapter 1. Quantitative Structure-Activity Relationship of Antibacterial Dodecylpyridinium Iodide Derivatives

1-1. Summary

N-Dodecylpyridinium iodide derivatives having hydroxyl, amino, methyl, chloro and/or trifluoromethyl groups on the pyridine ring were synthesized from the corresponding pyridines and *n*-dodecyl iodide under 80 MPa in order to delineate a quantitative structure-activity relationship. The bactericidal and bacterioclastic activity of the derivatives against *Escherichia coli* K12 W3110 was strongly affected by the kind of substituent groups these derivatives possessed and their positions. The electron-releasing groups such as amino and methyl groups markedly enhanced such activity, while the electron-attracting groups such as carboxyl and carbamoyl groups reduced it. The bactericidal activity of derivatives was dependent on acidic dissociation constant of the corresponding pyridines and the chemical shift of methylene protons adjacent to the pyridinium nitrogen. When bactericidal activity was plotted against bacterioclastic activity, the relationship was found to be linear. There was no correlation, however, between bactericidal activity and molecular hydrophobicity of derivatives. In conclusion, the findings suggest that the bactericidal activity of *N*-dodecylpyridinium derivatives is dependent on the electron density of the pyridinium nitrogen, and also on the bacterioclastic activity.

1-2. Materials and Methods

1-2-1. *Chemicals*

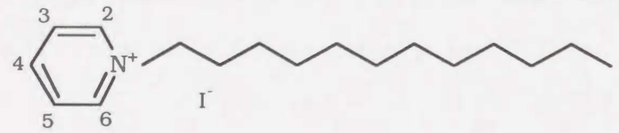
All chemicals for the synthesis of *N*-dodecylpyridinium derivatives were reagent grade commercial materials and used without further purification. Table 1-1 shows the chemical structures and abbreviations of the derivatives used in this study. The compounds (No. 1-17) were previously synthesized in our laboratory (Kourai et al., 1985b). Melting points were measured with a melting point apparatus (Mitamura Riken Kogyo Inc.) and are uncorrected. Elemental analyses were done with a Yanagimoto NT-5 elemental analysis apparatus. All the ^{1}H-NMR experiments were performed with a JEOL JEM-EX400 spectrometer at 400 MHz. Tetramethylsilane was used as an internal standard, and the chemical shift was presented as δ value.

1-2-2. *Synthesis of N-dodecylpyridinium derivatives*

The derivatives were prepared from corresponding pyridines: 3-hydroxypyridine, 2,3-diaminopyridine, 3,4-diaminopyridine, 2-amino-3-hydroxypyridine, 2-amino-4-methylpyridine, 2-amino-5-methylpyridine, 3-hydroxy-6-methylpyridine, 2-amino-5-chloropyridine, 2-amino-3,5-dichloropyridine, 2-amino-3-chloro-5-trifluoromethylpyridine, 3-(1-hydroxyethyl)pyridine, and 2-(2-hydroxyethyl) pyridine. A mixture of 0.1 mol of each pyridine and 0.1 mol of *n*-dodecyl iodide in 50 ml of absolute ethyl alcohol was heated at 80°C for 18 h under 80 MPa of static pressure with a high pressure

reactor (YHP-92, Yamashita Giken Co., Tokushima). After ethyl alcohol in the reaction mixture was removed with a rotary evaporator under a reduced pressure, the residue was recrystallized from ethyl alcohol-diethyl ether (1:10) to give an *N*-dodecylpyridinium derivative as white prisms.

Table 1-1. Chemical structures of dodecylpyridinium iodide derivatives.



No.	Abbrev.	Substituent groups				
		2	3	4	5	6
1	P-12	H	H	H	H	H
2	2M-12	CH_3	H	H	H	H
3	3M-12	H	CH_3	H	H	H
4	4M-12	H	H	CH_3	H	H
5	24DM-12	CH_3	H	CH_3	H	H
6	26DM-12	CH_3	H	H	H	CH_3
7	34DM-12	H	CH_3	CH_3	H	H
8	35DM-12	H	CH_3	H	CH_3	H
9	246TM-12	CH_3	H	CH_3	H	CH_3
10	3CABA-12	H	$CONH_2$	H	H	H
11	4CABA-12	H	H	$CONH_2$	H	H
12	2CABO-12	COOH	H	H	H	H
13	3CABO-12	H	COOH	H	H	H
14	4CABO-12	H	H	COOH	H	H
15	2A-12	NH_2	H	H	H	H
16	3A-12	H	NH_2	H	H	H
17	4A-12	H	H	NH_2	H	H

Table 1-1. (continued)

No.	Abbrev.	Substituent groups				
		2	3	4	5	6
18	3OH-12	H	OH	H	H	H
19	23DA-12	NH_2	NH_2	H	H	H
20	34DA-12	H	NH_2	NH_2	H	H
21	2A3OH-12	NH_2	OH	H	H	H
22	2A4M-12	NH_2	H	CH_3	H	H
23	2A5M-12	NH_2	H	H	CH_3	H
24	3OH6M-12	H	OH	H	H	CH_3
25	2A5C-12	NH_2	H	H	Cl	H
26	2A35DC-12	NH_2	Cl	H	Cl	H
27	2A3C5TF-12	NH_2	Cl	H	CF_3	H
28	31HE-12	H	$CH(OH)CH_3$	H	H	H
29	22HE-12	CH_2CH_2OH	H	H	H	H

1. *N*-dodecylpyridinium iodide, 2. *N*-dodecyl-2-methylpyridinium iodide, 3. *N*-dodecyl-3-methylpyridinium iodide, 4. *N*-dodecyl-4-methylpyridinium iodide, 5. *N*-dodecyl-2,4-dimethylpyridinium iodide, 6. *N*-dodecyl-2,6-dimethylpyridinium iodide, 7. *N*-dodecyl-3,4-dimethylpyridinium iodide, 8. *N*-dodecyl-3,5-dimethylpyridinium iodide, 9. *N*-dodecyl-2,4,6-trimethylpyridinium iodide, 10. *N*-dodecyl-3-carbamoylpyridinium iodide, 11. *N*-dodecyl-4-carbamoylpyridinium iodide, 12. *N*-dodecyl-2-carboxypyridinium iodide, 13. *N*-dodecyl-3-carboxypyridinium iodide, 14. *N*-dodecyl-4-carboxypyridinium iodide, 15. *N*-dodecyl-2-aminopyridinium iodide, 16. *N*-dodecyl-3-aminopyridinium iodide, 17. *N*-dodecyl-4-aminopyridinium iodide, 18. *N*-dodecyl-3-hydroxypyridinium iodide, 19. *N*-dodecyl-2,3-diaminopyridinium iodide, 20. *N*-dodecyl-3,4-diaminopyridinium iodide, 21. *N*-dodecyl-2-amino-3-hydroxypyridinium iodide, 22. *N*-dodecyl-2-amino-4-methylpyridinium iodide, 23. *N*-dodecyl-2-amino-5-methylpyridinium iodide, 24. *N*-dodecyl-3-hydroxy-6-methylpyridinium iodide, 25. *N*-dodecyl-2-amino-5-chloropyridinium iodide, 26. *N*-dodecyl-2-amino-3,5-dichloropyridinium iodide, 27. *N*-dodecyl-2-amino-3-chloro-5-trifluoromethylpyridinium iodide, 28. *N*-dodecyl-3-(1-hydroxyethyl)pyridinium iodide, 29. *N*-dodecyl-2-(2-hydroxyethyl)pyridinium iodide.

1-2-3. *Minimum bactericidal concentration (MBC)*

The MBCs were measured by a dilution method. The cell suspension (1 ml) of *E. coli* K12 W3110 preincubated in L-broth (Bacto tryptone 1 % (w/v), yeast extract 0.5 % (w/v), NaCl 0.5 % (w/v), pH 7.2) was inoculated into 100 ml of nutrient broth (Bacto beef extract 0.3 % (w/v), Bacto peptone 0.5 % (w/v), Difco Laboratories, Detroit, MI, U.S.A.). After incubation for 2 h at 37℃, the exponentially growing cells were harvested by centrifugation at 5000 × g for 10 min at 2℃, washed and suspended in sterilized ice-cooled water. The cell concentration of the suspension was adjusted to 4 × 10^6 cells per ml with ice-cooled water. One ml of an aqueous solution containing 1 mg of the tested QAC was diluted stepwise with sterilized water. Aliquots of 0.5 ml of the diluted solutions were mixed with 0.5 ml of cell suspension, and the mixtures were incubated in a water bath shaker for 30 min at 37℃. Then 0.1 ml aliquots of mixtures were taken out and inoculated into 2 ml of nutrient broth containing 1 % (v/v) polyoxyethylene sorbitan monooleate (Tween-80, Nacalai Tesque Inc., Kyoto). After the incubation at 37℃ for 24 h, MBC was determined by visual inspection.

1-2-4. *Scanning electron microscopy*

The exponential-phase cell suspension of *E. coli* K12 was treated using a P-12 (50 μM) at 37℃ for 120 s. The treated cells were collected with the membrane filter (pore size: 0.2 μm, Nuclepore,

Pleasanton, CA, U.S.A.) which was coated with a cationic polymer of trimethoxypropyldimethyloctadecyl ammonium chloride (Biosil, Dow Corning Asia, Tokyo). The cells on the filter were fixed with 3 % (v/v) glutaraldehyde solution for 1 h, dehydrated in a series of ethyl alcohol-distilled water mixture, and dried in a CO_2 atmosphere with a critical-point drier (HCP-2, HITACHI, Tokyo). The dried samples were mounted on the stage of the ion-coater of a Polaron E-1030, gold-coated for 3 min, and viewed with a scanning electron microscope (S-430, HITACHI, Tokyo).

1-2-5. *Critical vesiculation concentration (CVC)*

The CVC of the dodecylpyridinium derivative against exponential-phase cells of *E. coli* K12 was measured in terms of the increase in turbidity of the cell suspensions. Three ml of the cell suspension (OD_{660} = 0.3), which was kept in an ice bath, was preincubated at 37°C for 2 min. After the prescribed amount of aqueous solutions of the derivative were added to the suspension, the optical density of the suspension at 660 nm (OD_{660}) was measured continuously at 37°C for 200 s with a spectrophotometer (UV-160, Shimadzu Co., Kyoto) equipped with a TCC-240A, a thermo-electrically temperature-controlled cell holder. A plot of the turbidity increments (Δ OD_{660} /200 s) of the cell suspension vs. the concentration of the added derivative gave two straight lines. The concentration determined from the point of intersection of the two lines was defined as CVC (Kourai et al., 1994a).

1-2-6. *Acidic dissociation constant (pKa) of pyridines*

The pKa of the corresponding pyridine was measured by a pH-titration method (Brown et al., 1956) with 0.01 N hydrochloric acid at 25°C. The pKa data of other pyridines were quoted from our previous paper (Kourai et al., 1985b).

1-2-7. *Molecular hydrophobicity (R_M) of the pyridinium derivatives*

The R_M value, which can be determined by partition chromatography with reversed phases was used as a hydrophobicity parameter (Kourai et al., 1994c; 1995). The pyridinium derivative was applied to a reversed phase thin-layer chromatography plate (DC-Fertigplatten, RP-18 F_{254} S, Merck Co. Darmstadt, Germany) and chromatographed in an acetonitrile-ethyl alcohol-water (10:9:1) solvent system at 20°C. The flow rate of samples was determined under irradiation with UV light. The R_M value was calculated as follows:

$$R_M = \log (1/R_f - 1)$$

where R_f is the flow rate of the pyridinium derivative sample.

1-3. Results and Discussion

1-3-1. *Chemical properties of synthesized dodecylpyridinium derivatives*

Table 1-2 shows the data of elemental analysis, melting points and yields of synthesized QACs. The data for the elemental analysis are in fair agreement with their theoretical values. The NMR data for derivatives are shown in Table 1-3. The signals of 4.106-4.651 ppm (2H, t) and 0.892-0.895 ppm (3H, t) were assigned to the methylene protons of N^+-CH_2- and terminal methyl protons of -CH_3, respectively. The signals of 1.280-1.290 ppm (16H, m), 1.317-1.445 ppm (2H, m) and 1.794-2.026 ppm (2H, m) were assigned to the methylene protons of -$(CH_2)_{10}$-. These NMR data are consistent with the proposed structures of dodecylpyridinium derivatives. Reversed phase thin-layer chromatograms and high performance liquid chromatograms of the dodecylpyridinium derivatives also showed them to be in a high state of purity (data not shown).

1-3-2. *Bactericidal activity of dodecylpyridinium derivatives*

The antimicrobial activity of QACs is generally influenced by structural features, such as *N*-alkyl chain length (Daoud et al., 1983; Kourai et al., 1978; 1983c), and the number of quaternary nitrogen atoms (Kourai et al., 1986a). Kourai et al. (1985b) have reported the effect of substituent groups introduced into the pyridine ring on the antimicrobial activity of pyridinium derivatives. They used the derivatives (No. 1-17) listed in Table 1-1 and then revealed that the

Table 1-2. Chemical properties of dodecylpyridinium iodide derivatives.

No.	Iodide (abbrev.)	Elemental analysis						m.p. (°C)	Yield (%)
		C		H		N			
		Found	Calc.	Found	Calc.	Found	Calc.		
18	3OH-12	52.17	52.18	7.61	7.73	3.41	3.58	79-80	25.6
19	23DA-12	50.30	50.37	7.61	7.95	10.22	10.36	81-83	68.9
20	34DA-12	50.42	50.37	7.88	7.95	10.21	10.36	98-100	82.2
21	2A3OH-12	50.08	50.25	7.68	7.68	6.89	6.89	141-143	87.4
22	2A4M-12	53.46	53.46	8.01	8.23	6.71	6.93	59-60	85.6
23	2A5M-12	53.25	53.46	8.22	8.23	6.80	6.93	120-123	80.2
24	3OH6M-12	53.19	53.33	7.72	7.96	3.52	3.46	138-140	66.9
25	2A5C-12	48.07	48.07	6.99	7.12	6.50	6.59	109-111	68.2
26	2A35DC-12	44.28	44.46	6.10	6.36	5.93	6.10	52-55	67.5
27	2A3C5TF-12	43.84	43.87	6.01	5.93	5.26	5.68	59-60	55.6
28	31HE-12	54.30	54.41	8.07	8.17	3.45	3.34	44-46	65.3
29	22HE-12	54.63	54.41	7.91	8.17	3.29	3.34	170-172	67.1

Table 1-3. ^{1}H-NMR data of dodecylpyridinium iodide derivatives in CD_3OD.

No.	Iodide (abbrev.)	Chemical shift (δ ppm)
18	3OH-12	0.893(3H, t), 1.284(16H, m), 1.377(2H, m), 1.995(2H, m), 4.534(2H, t), 7.855(2H, d), 8.386-8.409(2H, m)
19	23DA-12	0.893(3H, t), 1.282(16H, m), 1.373(2H, m), 1.824(2H, m), 4.216(2H, t), 6.760-7.397(3H, m)
20	34DA-12	0.893(3H, t), 1.280(16H, m), 1.317(2H, m), 1.861(2H, m), 4.106(2H, t), 6.766(1H, d), 7.650(1H, d), 7.736(1H, dd)
21	2A3OH-12	0.892(3H, t), 1.281(16H, m), 1.374(2H, m), 1.821(2H, m), 4.210(2H, t), 6.775(1H, t), 7.153(1H, d), 7.496(1H, d)
22	2A4M-12	0.892(3H, t), 1.282(16H, m), 1.369(2H, m), 1.794(2H, m), 2.383(3H, s), 4.144(2H, t), 6.786(1H, dd), 6.886(1H, s), 7.862(1H, d)
23	2A5M-12	0.894(3H, t), 1.286(16H, m), 1.379(2H, m), 1.803(2H, m), 2.249(3H, s), 4.137(2H, t), 7.017(1H, d), 7.746(1H, dd), 7.831(1H, s)
24	3OH6M-12	0.894(3H, t), 1.290(16H, m), 1.439(2H, m), 1.926(2H, m), 2.741(3H, s), 4.489(2H, t), 7.745(1H, d), 7.792(1H, dd), 8.341(1H, d)
25	2A5C-12	0.895(3H, t), 1.290(16H, m), 1.384(2H, m), 1.811(2H, m), 4.151(2H, t), 7.092(1H, d), 7.890(1H, dd), 8.241(1H, d)
26	2A35DC-12	0.894(3H, t), 1.288(16H, m), 1.398(2H, m), 1.824(2H, m), 4.279(2H, t), 8.272(1H, d), 8.355(1H, d)
27	2A3C5TF-12	0.894(3H, t), 1.288(16H, m), 1.405(2H, m), 1.853(2H, m), 4.337(2H, t), 8.408(1H, d), 8.639(1H, d)
28	31HE-12	0.893(3H, t), 1.284(16H, m), 1.389(2H, m), 1.541 and 1.586(3H, dd), 2.026(2H, m), 4.651(2H, t), 5.093(1H, q), 8.071(1H, t), 8.582(1H, d), 8.902(1H, d), 9.022(1H, s)
29	22HE-12	0.893(3H, t), 1.288(16H, m), 1.445(2H, m), 1.984(2H, m), 2.513(2H, t), 3.485(2H, t), 4.617(2H, t), 7.544(1H, t), 7.688(1H, dd), 8.053(1H, t), 8.849(1H, dd)

introduction of electron-releasing groups, such as amino, methyl and propyl groups, at the 2-, 4- and/or 6-positions of the pyridine ring enhanced their bacteriostatic and antifungal activity, while the introduction of electron-attracting groups such as carboxyl and carbamoyl groups reduced it.

The MBC values of the QACs used in this study, including the derivatives (No. 1-17), were also affected by the kind of substituent groups and the introduced position in pyridine ring, as seen in Table 1-4. Almost all QACs having electron-releasing groups on the pyridine ring, exhibited high bactericidal activity (log MBC^{-1}). Especially *N*-dodecyl-3,4-diaminopyridinium iodide (34DA-12; No. 20) showed the highest activity of all and its MBC value was 10 μM. The introduction of two amino groups into pyridine ring resulted in an enhanced bactericidal activity of QACs. The activity of QACs (No.10-14) introduced electron-attracting groups, on the contrary, was highly reduced.

Further, the *p*-substituents introduced electron-releasing group were found to be more active than either the *o*- or *m*-compounds. Even the most case of di-substituted pyridinium compounds, the activity of the QACs having *p*-substituent group was higher than that of *o*- or *m*-substituted compounds. On the other hand, the *p*-carboxyl and *p*-carbamoyl groups, which are electron-attracting group, extremely decreased the bactericidal activity of QACs.

Table 1-4. Minimum bactericidal concentrations (MBC) and the critical vesiculation concentrations (CVC) of dodecylpyridinium iodide derivatives, acidic dissociation constants (pKa) of corresponding pyridines, ^{1}H-NMR data of methylene protons adjacent to the ammonium nitrogen of derivatives, and hydrophobicities.

No.	Pyridinium iodide (Abbrev.)	MBC (μM)	CVC (μM)	pKa[a]	δ (ppm) N^+- CH_2 -	Hydrophobicity (R_M)
1	P-12	71	26.9	5.19	4.677	-0.047
2	2M-12	112	26.3	5.97	4.603	-0.061
3	3M-12	112	30.2	5.68	4.603	-0.130
4	4M-12	72	18.6	6.02	4.597	-0.185
5	24DM-12	69	30.2	6.07	4.523	-0.163
6	26DM-12	87	12.3	6.75	4.517	-0.085
7	34DM-12	69	22.9	6.34	4.526	-0.178
8	35DM-12	69	28.8	5.88	4.537	-0.203
9	246TM-12	67	6.0	9.57	4.445	-0.226
10	3CABA-12	105	31.6	3.28	4.721	-0.290
11	4CABA-12	204	56.2	3.52	4.705	-0.341
12	2CABO-12	631	87.1	1.01	4.667	-0.169
13	3CABO-12	398	28.8	2.07	4.741	-0.059
14	4CABO-12	802	108.0	1.84	4.661	-0.169
15	2A-12	72	13.5	5.23	4.183	-0.395
16	3A-12	46	6.0	6.86	4.437	-0.321
17	4A-12	40	1.95	9.17	4.155	-0.325
18	3OH-12	155	39.8	4.69	4.534	-0.214
19	23DA-12	16	6.7	6.72	4.216	-0.242
20	34DA-12	10	4.3	8.90	4.106	-0.261
21	2A3OH-12	63	27.5	5.91	4.210	-0.286
22	2A4M-12	25	16.8	7.13	4.144	-0.194
23	2A5M-12	16	4.2	6.91	4.137	-0.194
24	3OH6M-12	63	9.5	5.35	4.489	-0.227
25	2A5C-12	25	21.5	4.61	4.151	-0.233
26	2A35DC-12	20	24.6	ND[b]	4.279	-0.244
27	2A3C5TF-12	78	13.4	ND	4.337	-0.290
28	31HE-12	39	21.6	4.93	4.651	-0.183
29	22HE-12	39	33.9	5.24	4.617	-0.106

a) The values of the pyridines (No.1-17) were quoted from the reference and the other values (No.18-29) were determined in water at 25℃ by the pH-titration method using 0.01 N hydrochloric acid.
b) Undetected

1-3-3. *Turbidity in the cell suspension*

The addition of QACs to the bacterial cell suspension rapidly caused turbidity in the suspension. The phenomenon of the increase in turbidity occurred at the concentrations below MBCs of QACs. The exponential-phase cells of *E. coli* K12 treated with P-12 (50 μM) for 120 s at 37°C formed blebs on their cell surfaces. Figure 1-1 shows the blebs (arrow a) developed on the end of the bacterial cell, and destruction (arrow b) of the cell surface structure. The blebs were very small (ca. 0.05-0.1 μm in diameter). Their size differed from that (ca. 0.2-0.3 μm) induced by heat treatment at 55°C for 10 min (Katsui et al., 1982), and that (0.01-0.03 μm) induced by polymyxin (Lounatmaa et al., 1976).

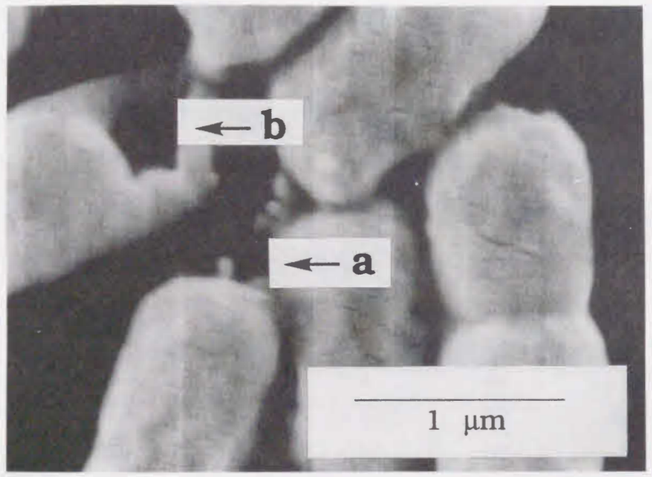


Fig. 1-1. Scanning electron micrograph of treated cell surfaces of *E. coli* K12 W3110 with *N*-dodecylpyridinium iodide (P-12). After the exponential-phase cells were treated with 50 μM of P-12 for 120 s at 37°C, cells were separated by filtration with a membrane filter (pore size, 0.2 μm), fixed in 3 % glutaraldehyde, washed, dried, and gold-coated. The arrow (a) indicates blebs. The arrow (b) indicates a destruction of cell surface structure.

Takasaki et al. (1994) also reported turbidity in a cell suspension of *Staphylococcus aureus* produced by a QAC, didecyldimethyl ammonium chloride (DDAC). They found the following phenomena caused by DDAC treatment: the formation of blebs on the cell surface, and leakage of intracellular components (K^+, 260 nm absorbing material, and phospholipid). From these findings, it was thought that the turbid materials consist of insoluble fragments of vesicles liberated from the bacterial cells.

An experimental result of turbidity produced by P-12 is illustrated in Fig. 1-2. The optical density (OD_{660}) of the cell suspensions of *E. coli* K12 with added P-12 reached a maximum during the first 60-120 s.

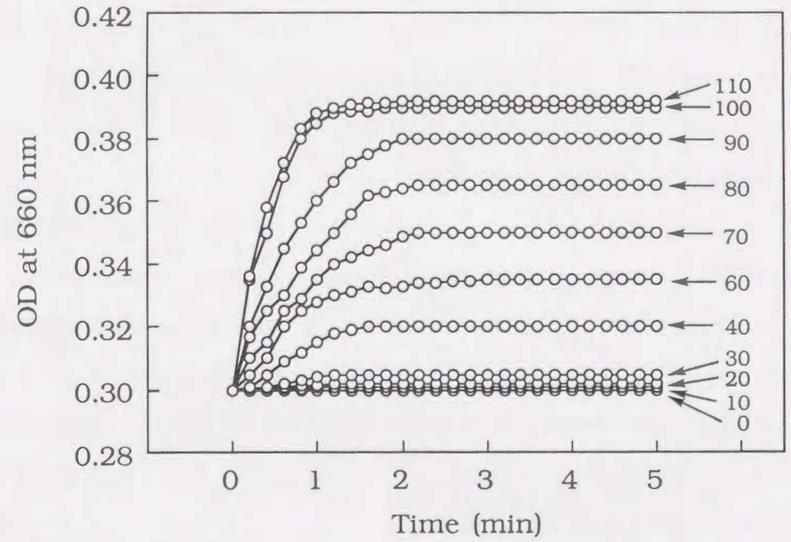


Fig. 1-2. Turbidity increase of the exponential-phase cell suspensions of *E. coli* K12 W3110 treated with P-12. The turbidity was measured at 37°C. The numerical values indicate the concentration (μM) of P-12.

The extent of the turbidity was related to the P-12 concentration. The increase (ΔOD_{660}/200 s) in turbidity was plotted against the concentration (10-100 μM) of the compound giving two straight lines as shown in Fig. 1-3. This result suggests that there is a critical concentration, like a critical micelle concentration, of the iodide needed to leak turbid material from bacterial cells. The concentration at the intersection was calculated from two regression equations obtained from the figure and this was defined as the CVC of the QAC. As seen in Fig. 1-3, the CVC of P-12 is 26.9 μM.

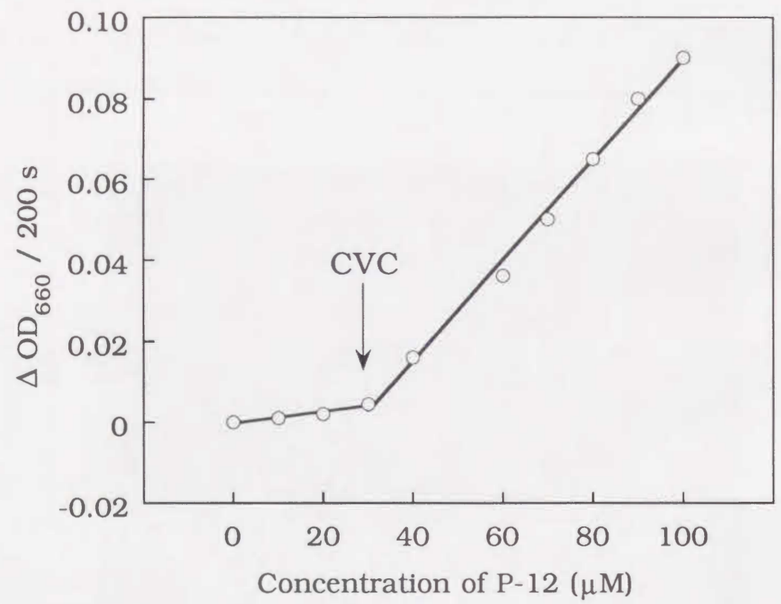


Fig. 1-3. Effect of the concentration of added P-12 on the turbidity increase (Δ OD at 660 nm for 200 s) of the cell suspensions. The arrow indicates the critical vesiculation concentration (CVC = 26.9 μM).

1-3-4. *Bacteroclastic activity of dodecylpyridinium derivatives*

The CVCs of the QACs used in this study are summarized in Table 1-4. Each value was also influenced by the kind of substituent groups and was below each MBC. The QACs having one or two amino groups relatively exhibited high bacterioclastic activity (log CVC^{-1}).

1-3-5. *Quantitative structure-activity relationship (QSAR) between bactericidal activity and the acidic dissociation constant*

Since the bactericidal activity of the synthesized QACs was remarkably affected by the kind of substituents and the positions on the pyridine ring, I conjectured that the activity is related to the electronic structure of the pyridinium molecule. The value of the acidic dissociation constant (pKa) of pyridine is directly proportional to the electron density of the nitrogen atom. Figure 1-4A shows the relation between the bactericidal activity of QACs against *E. coli* K12 and the pKa values for the corresponding pyridine. The activity increased linearly with an increase in the pKa value. When the activity was analyzed by a QSAR method using pKa at a 95 % confidence level, an equation was obtained. The correlation coefficient (*r*) was as high as 0.754. From this result, it was proved that the bactericidal activity of iodides having a dodecyl group against *E. coli* K12 is strikingly dependent on the acidic dissociation constant, that is, the electron density of the nitrogen atom.

1-3-6. *Relation between bactericidal activity and the chemical shift*

The electron density of pyridinium nitrogen of iodides can be also represented by a chemical shift (δ ppm) of methylene protons adjacent to ammonium nitrogen. A plot of the log MBC^{-1} versus δ ppm gave a straight line (Fig. 1-4B). It suggests that this equation obtained can be regarded as significant by the F-test, and the activity of iodides depends on the chemical shift. Consequently, the bactericidal activity of the pyridinium is closely connected with the electron density of the nitrogen atom.

1-3-7. *Relation between bactericidal activity and the molecular hydrophobicity*

Almost all QSAR studies have used exclusively experimental parameters, for example the critical micelle concentration (Xia et al., 1995) and partition coefficient (P). In 1973, Hansch and Clayton reported the QSAR between various drugs, including some QACs, and the molecular hydrophobicities (log P) calculated from the ratio of the concentrations of drugs in the aqueous and octanol phases.

In this study, R_M value was used instead of log P as a hydrophobicity parameter. It was determined easily by partition chromatography with reversed phases. The bactericidal activity of iodides against *E. coli* K12 was plotted against the R_M value. As seen in Fig. 1-4C, there was no correlation between them. It suggests that the bactericidal activity of iodides with a dodecyl group was scarcely

affected by molecular hydrophobicity. Devinsky et al. (1987) and Pavlikova-Moricka et al. (1994), however, demonstrated relationships between lipophilicity (hydrophobicity) and the antimicrobial activity of 4-alkyl-4-ethylmorpholinium bromides and bis-QAC derived from bis-(2-dimethylaminoethyl) glutarate which have different alkyl groups, using the QSAR method. Kourai et al. (1994c) have also reported that the bactericidal activity of *N*-alkyl-4-butenylpyridinium bromides (alkyl group; octyl, decyl, dodecyl, tetradecyl, hexadecyl and octadecyl) was correlated with their hydrophobicity. From these reports, it was thought that the molecular hydrophobicity of QAC mainly depends on the hydrophobicity of the alkyl group and the pyridinium skeleton of QAC. Therefore it suggests that hydrophobicity correlates with the bactericidal activity among the homologous series of QAC having various lengths of alkyl chains, but the bactericidal activity among various series of pyridinium derivatives having a dodecyl group, used in this study, is not dependent on the R_M value.

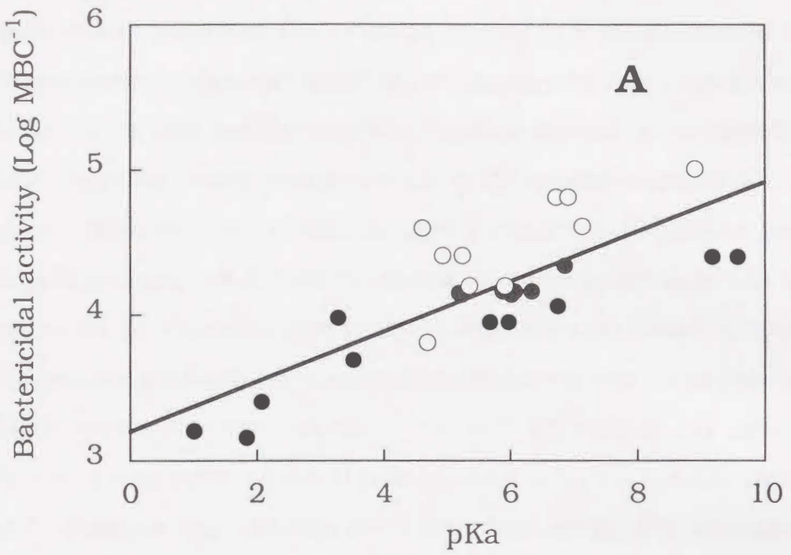


Fig. 1-4. (A)

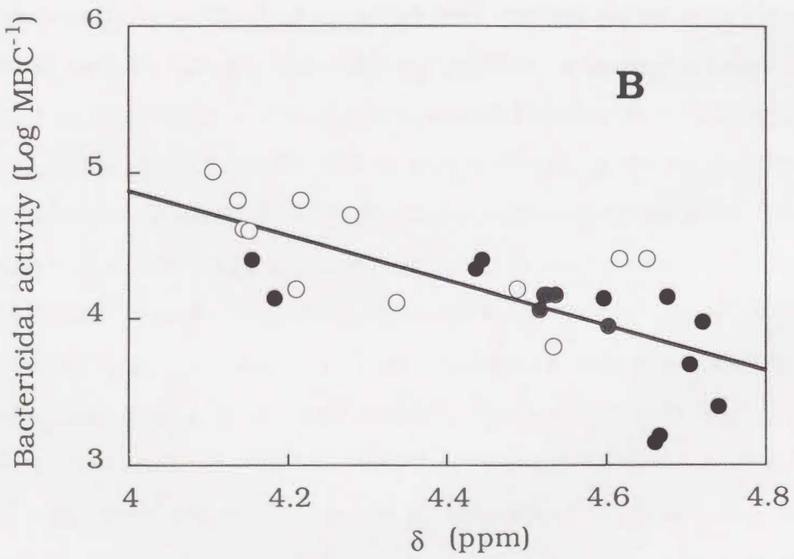


Fig. 1-4. (B)

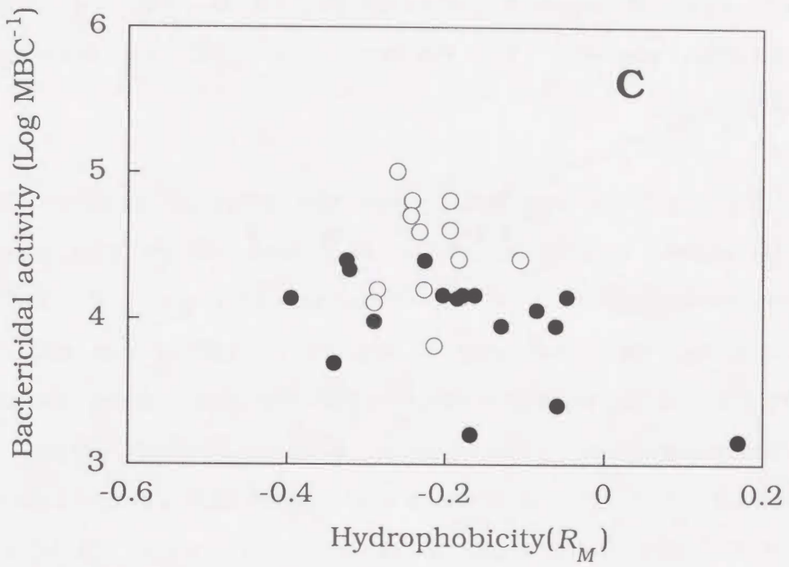


Fig. 1-4. (C)

Fig. 1-4. Relationship between the bactericidal activity (log MBC^{-1}) of *N*-dodecylpyridinium iodide derivative against exponentially growing cells of *E. coli* K12 W3110 and the acidic dissociation constant (pKa) of the corresponding pyridine (A), the chemical shift (δ ppm) of the methylene protons adjacent to ammonium nitrogen (B), and the hydrophobicity (R_M) of iodide (C). Bactericidal activity was defined as the logarithm of the reciprocal of the molar concentration. Symbols: ●, iodides (No. 1-17); ○, iodides (No. 18-29). Solid lines represent the equations: (A); Log MBC^{-1} = 0.16($\pm$0.06)pKa + 3.23($\pm$ 0.35), r = 0.754, F = 32.8 (Fo = 4.24 at 95 % confidence level), (B); Log MBC^{-1} = -1.53($\pm$0.57)ppm + 10.98($\pm$2.52), r = 0.730, F = 30.8 (Fo = 4.21).

1-3-8. *Relation between bacterioclastic activity and the acidic dissociation constant, the chemical shift and the molecular hydrophobicity*

Figures 1-5A and 1-5B show the relations between the bacterioclastic activity (log CVC^{-1}) of iodides and the pKa of the corresponding pyridines or the chemical shift (δ ppm) of iodides, respectively. Each plot gave a straight regression line and its correlation coefficient was 0.828 or 0.663. The results imply that the bacterioclastic activity as well as the bactericidal activity is dependent on the electron density of the pyridinium nitrogen atom.

On the other hand, a plot of bacterioclastic activity against the molecular hydrophobicity of iodides showed no correlation, as seen in Fig. 1-5C. It suggests that bacterioclastic activity is also scarcely affected by hydrophobicity.

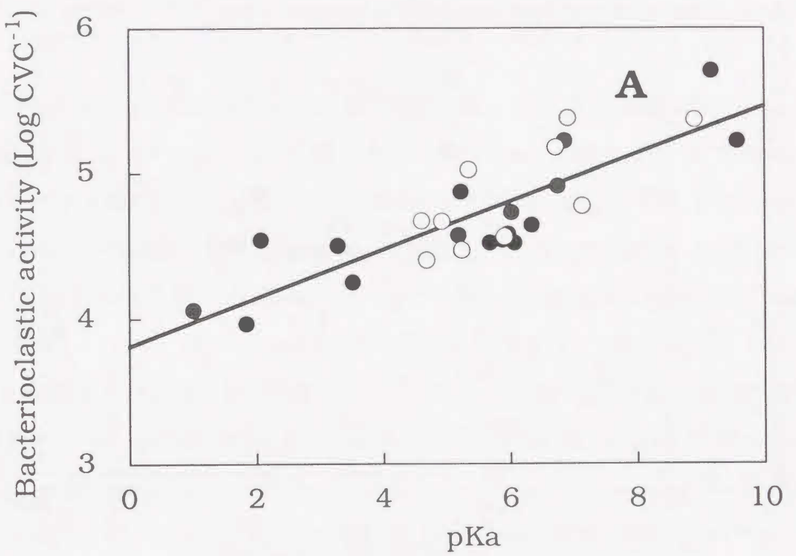


Fig. 1-5. (A)

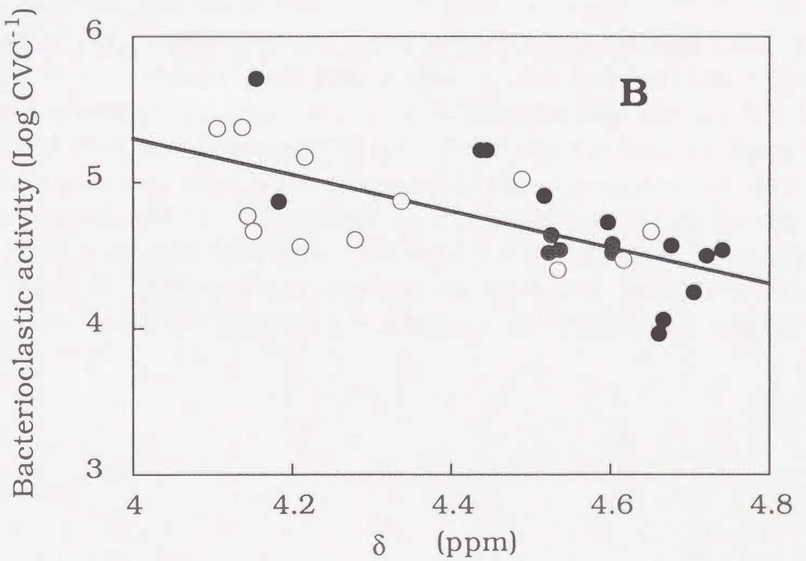


Fig. 1-5. (B)

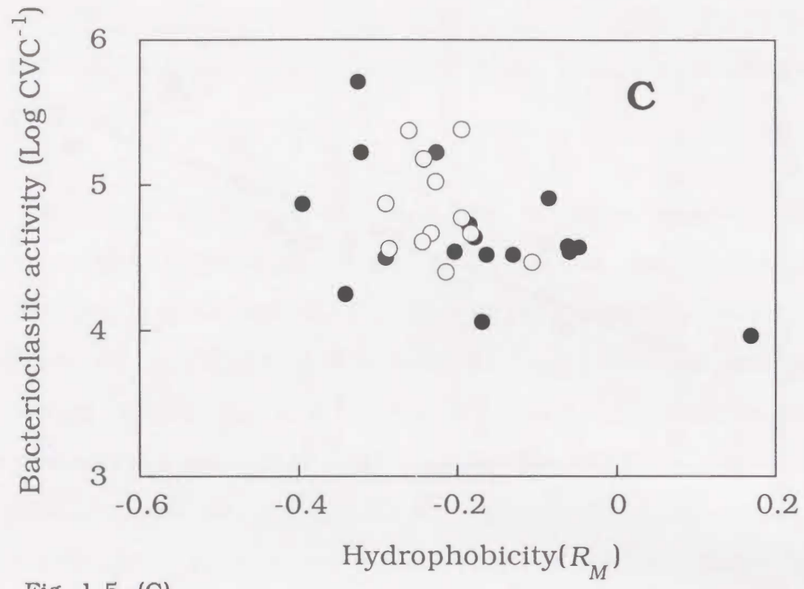


Fig. 1-5. (C)

Fig. 1-5. Relationship between the bacterioclastic activity (log CVC^{-1}) of dodecylpyridinium iodide derivative against *E. coli* K12 W3110 and the acidic dissociation constant (pKa) (A), chemical shift (δ ppm) (B), and hydrophobicity (R_M) (C). Bacterioclastic activity was defined as the logarithm of the reciprocal of the molar concentration. Symbols: ●, iodides (No. 1-17); ○, iodides (No. 18-29). Solid lines represent the equations: (A); Log CVC^{-1} = 0.17(±0.05)pKa + 3.81(± 0.27), r = 0.828, F = 54.7 (Fo = 4.24), (B); Log CVC^{-1} = -1.24(± 0.55)ppm + 10.27(±2.47), r = 0.663, F = 21.2 (Fo = 4.21).

1-3-9. *Relation between bactericidal activity and bacterioclastic activity*

Figure 1-6 shows the relation between bactericidal activity and bacterioclastic activity. The log MBC^{-1} was observed to increase linearly with the log CVC^{-1}. The coefficient (r) of the regression equation was 0.700. It suggests that the bactericidal activity of iodides against *E. coli* K12 is extremely dependent on bacterioclastic activity. From this result, the bacterioclastic action of iodide derivatives can be considered to be one stage of the bactericidal action. The bacterioclastic action is a combined action of blebbing and vesiculation on the bacterial cell surface. The bacterioclastic activity of iodides was influenced by the physicochemical parameters, pKa and chemical shift. These parameters reflect the electron density of the nitrogen atom. It is concluded that the *N*-dodecylpyridinium iodide derivative which has a high electron density on the nitrogen atom exhibits high bacterioclastic activity and bactericidal activity.

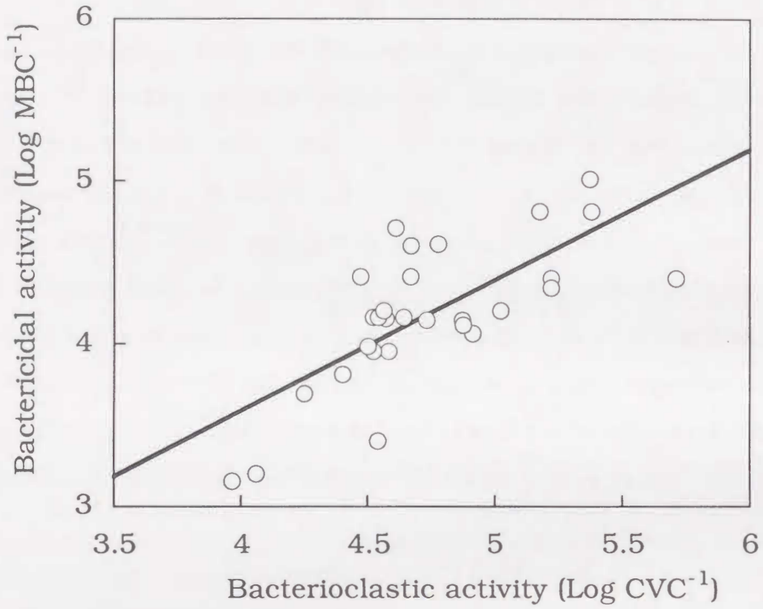


Fig. 1-6. Relationship between bactericidal activity (log MBC^{-1}) and the bacterioclastic activity (log CVC^{-1}) of *N*-dodecylpyridinium iodide derivative against *E. coli* K12 W3110. Solid line represents the equation: Log MBC^{-1} = 0.779(±0.316)Log CVC^{-1} + 0.479(±1.50), r = 0.700, F = 25.6 (Fo = 4.21).

Chapter 2. Effect of the Introduction of Benzylamino Group on Bactericidal Characteristics of QACs

2-1. Summary

N-Alkyl-2-benzylaminopyridinium iodides (2BAP-n), which have an electron-releasing group, were prepared from 2-benzylaminopyridine and *n*-alkyl iodide. These compounds could be expected to possess high bactericidal activity. 2BAP-12 exhibited a wide and high bactericidal spectrum and a higher activity than *N*-dodecylpyridinium iodide (P-12) having only alkyl group as a substituent on the pyridine ring against all bacteria tested. The bactericidal activity of 2BAP-n against *Escherichia coli* K12 W3110 was affected by the carbon number of the alkyl chain. The plot of the activity of 2BAP-n against molecular hydrophobicity was found to be parabolic. From the result, it was suggested that the activity of 2BAP-n is dependent on the molecular hydrophobicity. Similarly, the activity had a linear dependence to the hydrophobicity of cell surfaces. The activity increased with an increase in the hydrophobicity, as well as that of general QACs. 2BAP-12 maintained high activity in the pH range from 5 to 8.5, though the activity was lower than that of P-12 in alkaline solution. It was thought that this exceptional bactericidal property against pH is due to the effect of big substituent, benzylamino group, introduced into the pyridine ring.

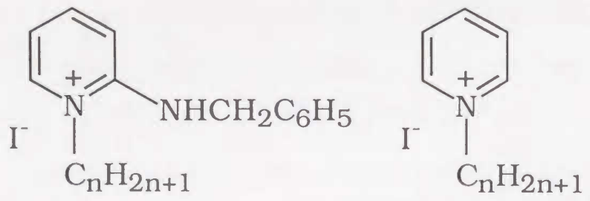
2-2. Materials and Methods

2-2-1. *Chemicals*

All chemicals for the synthesis of *N*-alkyl-2-benzylaminopyridinium iodides (alkyl group: hexyl, octyl, decyl, dodecyl, tetradecyl, hexadecyl and octadecyl) were reagent grade commercial materials and used without further purification. The iodides are abbreviated as 2BAP-n, with "n" indicating the carbon number of the alkyl chain. *N*-Alkylpyridinium iodides (P-n) were prepared in our laboratory (Kourai et al., 1980a) and used in order to make a comparison with 2BAP-n.

2-2-2. *Synthesis of N-alkyl-2-benzylaminopyridinium iodides*

Figure 2-1 shows the chemical structure of synthesized 2BAP-n. 2-Benzylaminopyridine was prepared in 90 % yield by dehydration of



2BAP-n P-n

Fig. 2-1. Chemical structures of *N*-alkyl-2-benzylaminopyridinium iodide (2BAP-n) and *N*-alkylpyridinium iodide (P-n). The abbreviation, n, indicates the carbon numbers of alkyl chain, and n is 6, 8, 10, 12, 14, 16 or 18.

2-aminopyridine and benzylalcohol with KOH, according to the method of Sprinzak (1958).

A mixture of 2-benzylaminopyridine (1.0 mol), *n*-alkyl iodide (1.0 mol) and ethyl alcohol was refluxed at 80℃ for 48 h under 80 MPa of static pressure. After the ethyl alcohol in the reaction mixture was removed with a rotary vacuum evaporator, diethyl ether (100 ml) was added to the residue to give coarse crystals. The crystals were recrystallized from ethyl alcohol-ethyl acetate (1:4).

2-2-3. *Molecular hydrophobicity (log P)*

The partition coefficients (P) of 2BAP-n between *n*-octylalcohol and water were regarded as representing their hydrophobicity. To 100 ml flasks, each containing 25 ml of the QAC aqueous solution (0.02-0.16 % (w/v)), 25 ml portions of *n*-octylalcohol were added respectively. The flasks were shaken in a water bath equipped with a reciprocal shaker at 70 strokes/min for 24 h at 37℃, and allowed to stand for 10 min in the bath, then the organic and aqueous layers were taken out separately. The amounts of the QAC in them were determined on a spectrophotometer at 220 nm. The hydrophobicity (log P) were given by

$$n \log C_a + \log P = \log C_b$$

where C_a was the concentration of a particular iodide in the aqueous layer, C_b that in the organic layer, and n the association coefficient. When $\log C_a = 0$, the molecular hydrophobicity was given by $\log P = \log C_b$.

The data of P-n were quoted from the previous paper (Kourai et al., 1983b).

2-2-4. *Hydrophobicity of cell surfaces*

The hydrophobicity of the bacterial cell surface of *E. coli* K12 W3110 was determined using the partition system of *n*-hexadecane-physiological saline, as described by Kourai et al. (1989). The hydrophobicities of the other bacteria listed in Table 2-2 were quoted from the same reference. To the 100 ml flasks containing 5 ml exponential-phase cell suspensions (OD_{500} = 0.05−0.4), 5 ml *n*-hexadecane was added. The flasks were shaken in a water-bath at 37℃ for 10 min at 50 strokes/min. Then the absorbance of the aqueous phase was measured with a spectrophotometer at 500 nm. The hydrophobicity index (*HI*) is given as

$$(C_I\text{-}C_A) \,/\, C_A{}^n = HI$$

where C_I and C_A are the concentrations of bacterium in the aqueous phases, before and after partitioning, $(C_I\text{-}C_A)$ is the concentration of cells moved and n is the association constant.

$$n \log C_A + \log HI = \log (C_I\text{-}C_A)$$

When $\log C_A = 0$, the hydrophobicity ($\log HI$) is given as

$$\log HI = \log (C_I\text{-}C_A)$$

2-3. Results and Discussion

2-3-1. *Chemical structures and properties of synthesized 2BAP-n*

Table 2-1 shows the analytical data and yields of the synthesized 2BAP-n. The data for the elemental analysis are in fair agreement with their theoretical values. The following NMR data (δ) for 2BAP-12 is given as a typical result. The signals of 4.35 ppm (2H, t) and 0.89 ppm (3H, t) were assigned to the methylene protons of $N^+\text{-}CH_2\text{-}$ and terminal methyl protons of $\text{-}CH_3$, respectively. The signals of 6.96 ppm (1H, m), 7.09 ppm (1H, d), 7.90 ppm (1H, m) and 8.14 ppm (1H, m) were assigned to the aromatic protons of *o*-substituted pyridine ring. The signals of 4.74 ppm (2H, s) and 7.35-7.41 ppm (5H, m) were assigned to the methylene protons of $C_6H_5\text{-}CH_2\text{-}$ and the aromatic protons of benzene ring, respectively. These NMR data are consistent with the proposed structure of 2BAP-12. The NMR data of other iodides are also consistent with their structures (data not shown).

Table 2-1. Physical properties of *N*-alkyl-2-benzylaminopyridinium iodides (2BAP-n).

Iodide (abbrev.)	Elemental analysis (%)						m.p. (℃)	Yield (%)
	C		H		N			
	Found	Calc.	Found	Calc.	Found	Calc.		
2BAP-6	54.31	54.55	6.24	6.36	7.04	7.07	126-127	42.0
2BAP-8	56.67	56.60	6.81	6.89	6.58	6.60	167-170	35.0
2BAP-10	58.48	58.41	7.17	7.35	6.40	6.19	98-100	32.0
2BAP-12	59.80	60.00	7.56	7.76	6.05	5.83	104-107	20.0
2BAP-14	61.17	61.40	7.84	8.13	5.34	5.51	109-110	18.0
2BAP-16	62.38	62.68	8.20	8.45	5.00	5.22	112-113	22.0
2BAP-18	63.52	63.81	8.52	8.75	4.91	4.96	108-110	45.0

2-3-2. *Bactericidal activity*

Table 2-2 shows the MBCs of 2BAP-12 and P-12 against exponential-phase cells of gram-negative bacteria (No. 1-7) and gram-positive bacteria (No. 8-12). 2BAP-12 exhibited a wide and high bactericidal spectrum and the activity was higher than that of P-12 against all bacteria tested. The MBC values of 2BAP-12 against gram-positive bacteria, especially against *B. subtilis* (No. 8) and *S. aureus* (No. 11 and 12), were very small.

In addition, the activity (MBC = 41.0 μM) against *E. coli* K12 W3110 was stronger than that (MBC = 72.0 μM) of *N*-dodecyl-2-aminopyridinium iodide (2A-12) having amino group on pyridine ring, listed in Table 1-4 (Chapter 1). From these results, I presumed that the introduction of benzylamino group into *o*-position of the pyridine ring enhanced the bactericidal activity against bacteria.

Table 2-2. Minimum bactericidal concentrations (MBCs) of 2BAP-12 and P-12 against exponential-phase cells of gram-negative and gram-positive bacteria.

No.	Strain	MBC(μM)[a]	
		2BAP-12[b]	P-12[C]
1	*Pseudomonas aeruginosa* ATCC 27583	400	1000
2	*Pseudomonas aeruginosa* ATCC 10145	320	640
3	*Klebsiella pneumoniae* ATCC 13883	65.5	205
4	*Proteus rettgeri* NIH 96	52.4	200
5	*Proteus mirabilis* IFO 3849	131	250
6	*Escherichia coli* K12 OUT 8401	33.6	65.5
7	*Escherichia coli* K12 W3110	41.0	83.2
8	*Bacillus subtilis* IFO 3134	13.4	13.4
9	*Bacillus megaterium* IFO 3003	21.0	80.0
10	*Bacillus subtilis* var. *niger* OUT 4380	41.0	81.9
11	*Staphylococcus aureus* IFO 12732	13.4	13.4
12	*Staphylococcus aureus* ATCC 25923	12.5	12.5

a) MBCs were measured by a dilution method at 30℃ for 30 min.
b) *N*-dodecyl-2-benzylaminopyridinium iodide.
c) *N*-dodecylpyridinium iodide.

2-3-3. *Molecular hydrophobicity*

In order to investigate the influence of molecular hydrophobicity of 2BAP-n on the bactericidal activity, MBCs of all compounds synthesized were measured and compared with those of P-n. In almost case, the lengthening of the chain of the alkyl substituent brings increase in molecular hydrophobicity. As it is supposed that the first step of the bactericidal action of QACs, including 2BAP-n and P-n, is a hydrophobic interaction between the molecules and cell surfaces, the molecular hydrophobicity would be an important factor to appear a strong bactericidal activity. As seen in Fig. 2-2,

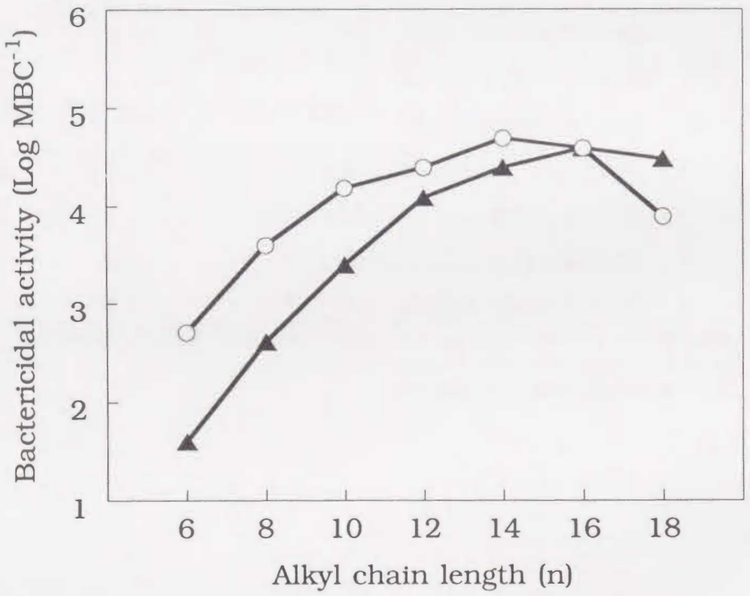


Fig. 2-2. Effect of alkyl chain length (n) on the bactericidal activity (log MBC^{-1}) of 2BAP-n (○) and P-n (▲) against exponential-phase cells of *E. coli* K12 W3110. MBCs were measured by a dilution method at 30℃ for 30 min.

the activity of 2BAP-n increased with increasing molecular hydrophobicity and reached a maximum with C14. In the case of P-n, the highest activity was shown by P-16.

When the molecular hydrophobicity is expressed as log P, where P is the *n*-octylalcohol-water partition coefficient, the plots of the bactericidal activity of 2BAP-n and P-n against log P were found to be parabolic (Fig. 2-3). From the figure, it is clear that 2BAP-14 molecule is more hydrophobic than P-16. Each homologous series of QAC had the most suitable hydrophobicity to exhibit the highest bactericidal activity against *E. coli* K12 W3110. The hydrophobicity with optimal activity would vary with the tested bacteria, reflecting the differences in their membrane structure.

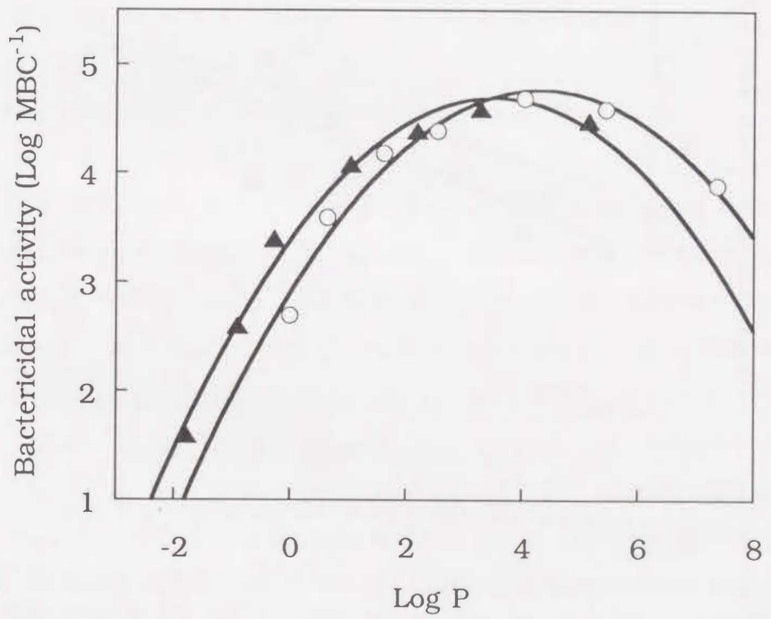


Fig. 2-3. Relationship between bactericidal activity (log MBC^{-1}) and molecular hydrophobicity (log P) of 2BAP-n (○) and P-n (▲) against exponential-phase cells of *E. coli* K12 W3110.

2-3-4. *Relationship between the bactericidal activity and the hydrophobicity of the cell surfaces*

In general, QACs have a higher bactericidal activity against gram-positive bacteria than against gram-negative bacteria (Kourai et al., 1989). The reason is that there is a hydrophobic interaction between QAC molecules and the bacterial cell surfaces, and one stage of bactericidal action occurs on the cell surfaces. The bactericidal activity of most QACs therefore tends to depend on the hydrophobicity of cell surfaces. Figure 2-4 shows the relationship

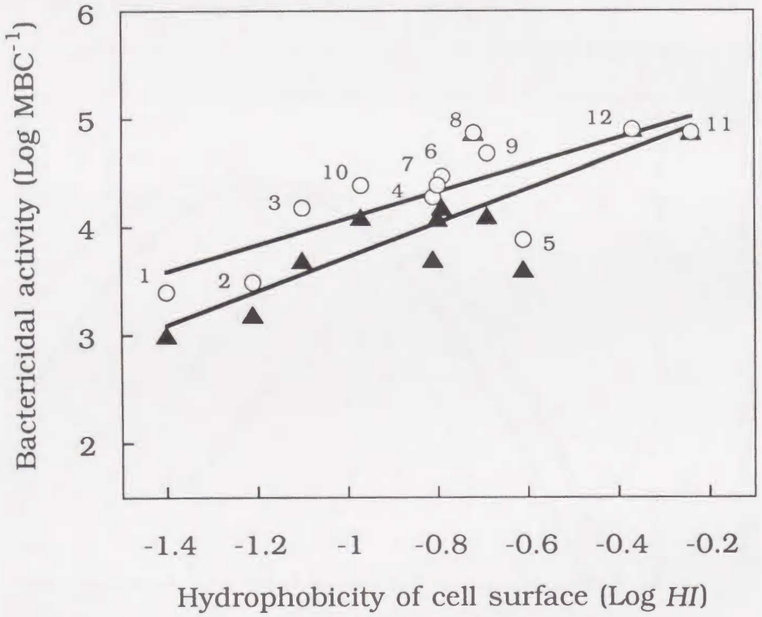


Fig. 2-4. Relationship between bactericidal activity (log MBC^{-1}) of 2BAP-n (○) and P-n (▲) and hydrophobicity (log *HI*) of exponential-phase cell surface. The hydrophobicity was determined using the partition system of *n*-hexadecane-physiological saline. The number beside each datum point indicates the bacterial species stated in Table 2-2.

between the bactericidal activity of 2BAP-12 and P-12 and the hydrophobicity (log *HI*) of the bacterial cell surfaces. The activity of both compounds increased linearly with an increase in the hydrophobicity. In other words, the QACs are more effective against the bacteria having hydrophobic surfaces, such as *S. aureus*, than the bacteria having hydrophilic surfaces, such as *P. aeruginosa*. The relations are expressed by the equation, log MBC^{-1} = a + b log *HI*. The coefficients a and b were statistically determined by the least-squares method at 95 % confidence level; 2BAP-n: a = 5.32, b = 1.24, r = 0.801, P-n: a = 5.30, b = 1.58, r = 0.828. From these data, the equations can be regarded as significant. Therefore, it is concluded that the bactericidal activity of 2BAP-12 and P-12 are dependent on the hydrophobicity of cell surface, as well as general QACs.

2-3-5. *Effect of pH on bactericidal activity*

The MBCs of 2BAP-12 and P-12 were measured using 0.05 M phosphate buffer (pH: 5, 6, 7, 8 and 8.5). As the bactericidal activity of QACs are influenced by salts such as NaCl and KCl in solution, we experimentally determined that the concentration of phosphate in the buffer had a negligible influence on the activity. As shown in Fig. 2-5, 2BAP-12 maintained high activity in the pH range from 5 to 8.5, though the activity was lower than that of P-12 in alkaline solution. The activity of P-12 was strongly affected by the pH. Thus, QACs such as P-12 are generally more effective in alkaline solution than in acidic solution. We also reported the bactericidal activity of *N*-dodecylcyanopyridinium bromides which have a cyano group at the 2-, 3-, or 4-position of the pyridine ring were strongly dependent

upon the pH value of the test solution (Maeda et al., 1996). This is one of the disadvantages of QACs. However, 2BAP-12 showed a low dependence of the bactericidal activity on pH. This result could be explained by the effect of benzylamino group, which is relatively big substituent, introduced into the pyridine ring.

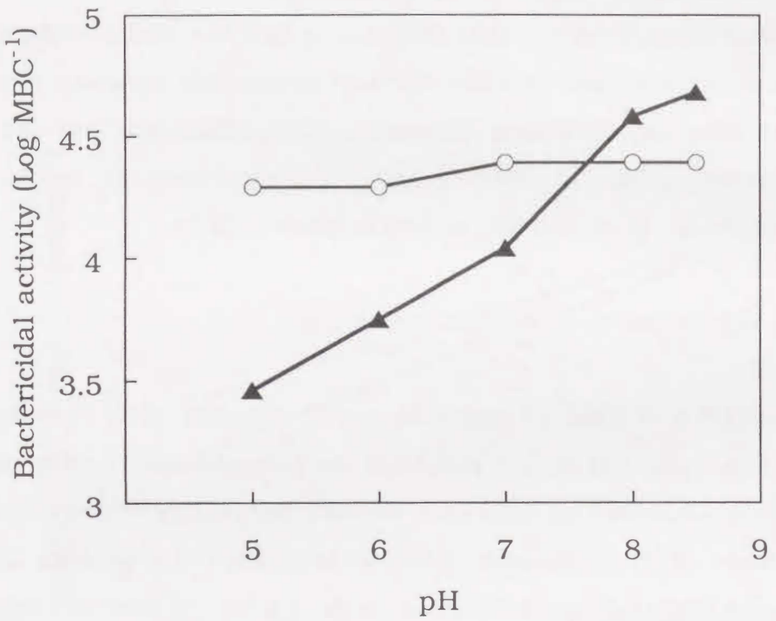


Fig. 2-5. Effect of pH on the bactericidal activity (log MBC^{-1}) of 2BAP-12 (○) and P-12 (▲) against exponential-phase cells of *E. coli* K12 W3110. MBCs were measured using 0.05 M phosphate buffer.

Chapter 3. Effect of the Introduction of Unsaturated Substituent into Pyridine Ring on Antibacterial Activity

3-1. Summary

New QACs, *N*-alkyl-4-allylthiopyridinium bromides (ATP-n), introduced an unsaturated substituent into pyridine ring were synthesized. 4-Allylthiopyridine which was synthesized from 4-mercaptopyridine and 3-bromopropene was allowed to react with *n*-alkyl bromides under 80 MPa of static pressure. ATP-n exhibited a strong bactericidal activity, compared with *N*-alkyl-4-butenylpyridinium bromides (BNP-n) having butenyl group on the pyridine ring and *N*-alkylpyridinium bromides (PBr-n). The bacteriostatic activity of ATP-n with long alkyl chain (n = 14-18), however, was lower than those of the corresponding BNP-n. The effect of the allylthio group on the bacterioclastic activity was higher than that of the butenyl group.

3-2. Materials and Methods

3-2-1. *Chemicals*

All chemicals for the synthesis of ATP-n were reagent grade commercial materials and used without further purification. *N*-Alkyl-4-butenylpyridinium bromides (BNP-n) were previously synthesized in our laboratory (Kourai et al., 1994c).

3-2-2. *Synthesis of N-alkyl-4-allylthiopyridinium bromides*

A mixture of 0.05 mol of 4-mercaptopyridine, 0.05 mol of 3-bromopropene and 20 ml of ethyl alcohol was heated under reflux for 24 h. The reaction mixture was made strongly basic to litmus with 0.1 N sodium hydroxide and extracted with diethyl ether. Distillation of the ether solution gave 4-allylthiopyridine a 92.8 % yield. A mixture of 0.05 mol of 4-allylthiopyridine and 0.05 mol of *n*-alkyl bromide (alkyl group: decyl, dodecyl, tetradecyl, hexadecyl and octadecyl) in 20 ml of ethyl alcohol was heated at 80°C for 72 h under 80 MPa of static pressure. After the ethyl alcohol in the reaction mixture was removed with a rotary evaporator under a reduced pressure at 80°C, the residue was dissolved in 200 ml of diethyl ether to give coarse crystals at -20°C. The coarse crystals were recrystallized from ethyl alcohol-diethyl ether (1:1) to give ATP-n.

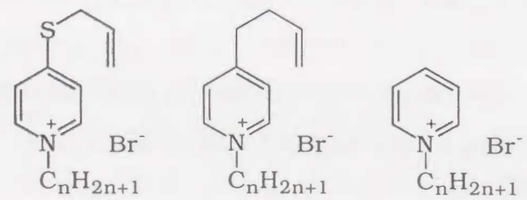
3-2-3. *Minimum inhibitory concentration (MIC)*

The MICs against bacteria were measured by a broth dilution method. A nutrient broth (Bacto beef extract 0.3 % (w/v), Bacto peptone 0.5 % (w/v), Difco Laboratories, Detroit, MI, U.S.A.) was used for the antibacterial test. Ten ml of the broth containing 10 mg of each tested compound was diluted stepwise with the broth. The bacterium was preincubated in L-broth (Bacto tryptone 1 % (w/v), yeast extract 0.5 % (w/v), NaCl 0.5 % (w/v), pH 7.2) for 18 h at 37℃. A 1 ml aliquot of the culture was inoculated into 500 ml of nutrient broth, then 2 ml portions of the inoculated broth were pipetted into sterilized test tubes each containing 2 ml of diluted QAC. The mixture was incubated at 37℃ for 24 h, and the MICs were determined by visual inspection.

3-3. Results and Discussion

3-3-1. *Chemical properties of ATP-n*

Figure 3-1 shows the chemical structures of ATP-n, BNP-n and PBr-n. BNP-n and PBr-n were used in order to investigate the effect of the allylthio group of ATP-n on the antibacterial activity.



ATP-n BNP-n PBr-n

Fig. 3-1. Chemical structures of *N*-alkyl-4-allylthiopyridinium bromide (ATP-n), *N*-alkyl-4-butenylpyridinium bromide (BNP-n) and *N*-alkylpyridinium bromide (PBr-n).

Table 3-1 shows the analytical data and yields of the synthesized ATP-n. The data of elemental analysis are in fair agreement with their theoretical values. The ^{1}H-NMR data (δ) for ATP-12 in CD_3OD is as follows; 0.89 (3H, t, *J*=6.1), 1.28 (16H, m), 1.35 (2H, m), 1.95 (2H, m), 3.98 (2H, d, *J*=6.4), 4.45 (2H, t, *J*=7.3), 5.31 (1H, d, *J*=10.3), 5.50 (1H, d, *J*=17.1), 5.94 (1H, m), 7.84 (2H, d, *J*=5.9), 8.59 (2H, d, *J*=5.4). The signals of 4.45 ppm were assigned to the methylene protons of N^+-CH_2-. The signals of 5.31 ppm, 5.50 ppm and 5.94 ppm were assigned to the vinyl protons of CH_2=CH-. The signals of 7.84 ppm and 8.59 ppm were assigned to the aromatic protons of *p*-substituted pyridine

ring. This NMR data is consistent with the proposed structure of ATP-12. The NMR data of other bromides are also consistent with their structures (data not shown).

Table 3-1. Chemical properties and yields of synthesized *N*-alkyl-4-allylthiopyridinium bromides (ATP-n).

Bromide (abbrev.)	Elemental analysis (%)						m.p. (°C)	Yield (%)
	C		H		N			
	Found	Calc.	Found	Calc.	Found	Calc.		
ATP-10	57.89	58.05	8.03	8.12	3.56	3.76	49.1-51.2	24.5
ATP-12	59.72	59.99	8.36	8.56	3.50	3.50	68.0-70.1	9.4
ATP-14	60.97	61.66	8.84	8.93	3.27	3.25	85.1-87.6	5.0
ATP-16	63.10	63.14	9.19	9.27	3.07	3.08	92.2-94.3	7.7
ATP-18	64.24	64.44	9.45	9.57	2.90	2.89	96.3-98.9	17.5

3-3-2. *Antibacterial activity*

The effects of alkyl chain length of ATP-n, BNP-n and PBr-n on the antibacterial activity were investigated. In general, the bactericidal activity of QACs was strikingly affected by the length of the alkyl chain attached to the ammonium nitrogen, as described in Chapter 2 (2-3-3.). The activity of ATP-n was also influenced by the alkyl chain length (Fig. 3-2A). The activity of ATP-n (n = 10-16) increased with the lengthening of the chain of *N*-alkyl substituent, but the activity of ATP-18 did not. All ATP-n exhibited stronger bactericidal activity than BNP-n and PBr-n. The result indicates that the introduction of allylthio group into the *p*-position of pyridine ring enhances the bactericidal activity of pyridinium salt derivatives.

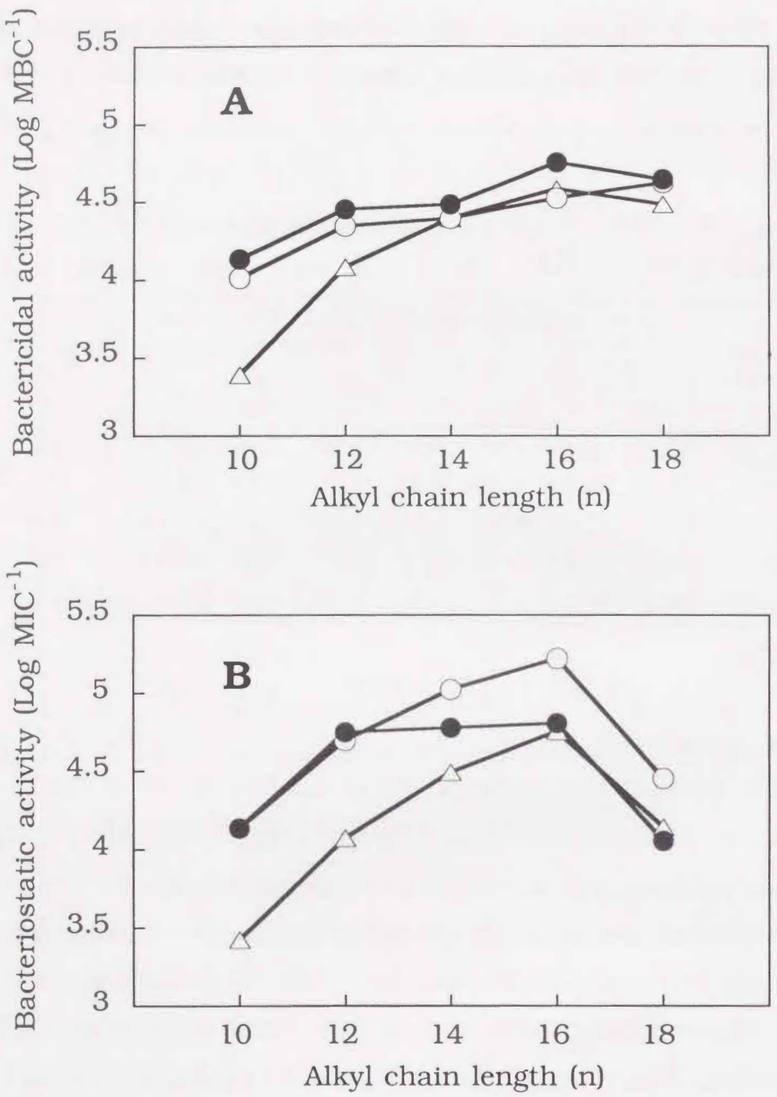


Fig. 3-2. Comparison of bactericidal activity (log MBC^{-1}) (A) and bacteriostatic activity (log MIC^{-1}) (B) of ATP-n (●), BNP-n (○) and PBr-n (△) against *E. coli* K12 W3110. Bactericidal activity and bacteriostatic activity were defined as the logarithm of the reciprocal of the molar concentration.

Similarly the bacteriostatic activity of three series of bromides was affected by the carbon number of alkyl chain, as seen in Fig. 3-2B. Contrary to the above result (Fig. 3-2A), ATP-14, ATP-16 and ATP-18 exhibited lower bacteriostatic activity than BNP-14, BNP-16 and BNP-18, respectively. It may be due to the difference between MBC and MIC measurement systems. The contact time for the bacterial cells and the bromides for MIC measurement is 24 h, but the time for MBC is 30 min. All BNP-n, except for BNP-18, exhibited very high bacteriostatic activity. This suggests that the antibacterial activity of BNP-n is extremely affected by the contact time, compared with that of ATP-n and PBr-n. On the other hand, all bromides with octadecyl group (ATP-18, BNP-18 and PBr-18) exhibited low activity. This was due to the influence of the medium components in the MIC measurement system. As the bromides have long alkyl chains in the molecules, the hydrophobicities of the molecules are very high. It is thought that a hydrophobic association between the bromides and medium components caused the decrease in the apparent concentration of bromides in the MIC measurement system.

3-3-3. *Antibacterial spectrum*

Table 3-2 shows the MICs of ATP-12 and BNP-12 against gram-negative bacteria (8 strains) and gram-positive bacteria (7 strains). ATP-12 exhibited a wide and strong antibacterial spectrum against all bacteria tested in this study. The bacteriostatic activity (MIC = 0.61 μM) of ATP-12 against *S. aureus* is higher than that (MIC = 28 μM) of 3,3',5,5'-tetrachloro-2,2'-dihydroxydiphenyl sulfide (Bithionol), which is a representative of sulfide (Horiguchi, 1982). The activity of

Table 3-2. Minimum inhibitory concentrations (MICs) of *N*-dodecyl-4-allylthiopyridinium bromide (ATP-12) and *N*-dodecyl-4-butenyl-pyridinium bromide (BNP-12) against stationary-phase cells of gram-negative and gram-positive bacteria.

Strain	MIC (μM)	
	ATP-12	BNP-12
Gram-negative bacteria		
Pseudomonas aeruginosa ATCC 10145	39.1	39.1
Pseudomonas aeruginosa IFO 3080	39.1	78.1
Klebsiella pneumoniae ATCC 4352	2.44	4.88
Klebsiella pneumoniae ATCC 13883	19.5	19.5
Proteus rettgeri NIH 96	6.50	20.4
Proteus mirabilis IFO 3849	78.1	156
Escherichia coli K12 OUT 8401	4.88	4.88
Escherichia coli K12 W3110	19.5	20.4
Gram-positive bacteria		
Bacillus subtilis ATCC 6633	1.22	2.56
Bacillus subtilis IFO 3134	1.22	1.22
Bacillus cereus IFO 3001	2.44	2.44
Bacillus megaterium IFO 3003	1.22	1.22
Staphylococcus aureus IFO 12732	0.61	5.12
Staphylococcus aureus JC1 (MRSA)	4.88	4.88
Micrococcus luteus IFO 12708	1.22	1.22

ATP-12, however, was approximately equal to that of BNP-12. It is considered that the effect of allylthio group on the pyridine ring on the bacteriostatic activity is as high as that of the butenyl group.

On the other hand, both bromides similarly showed stronger activity against gram-positive bacteria than against gram-negative bacteria. The cell surfaces of gram-positive bacteria are more hydrophobic than those of gram-negative bacteria, as seen in Fig. 2-4 (Chapter 2). It was suggested that there was a hydrophobic interaction between the bromides (ATP-12 and BNP-12) and the

bacterial cell surfaces, and that one of the stages of bactericidal action occurred on the cell surfaces.

3-3-4. *Effect of temperature on bactericidal activity*

To investigate the interaction between the bacterial cell surfaces and the bromides, the MBCs of ATP-12, BNP-12 and PBr-12 against exponential-phase cells of *E. coli* K12 W3110 were measured at 10, 20, 30, 35, 37 and 40℃. In general, increasing temperature tends to increase the bactericidal activity of QACs. As seen in Fig. 3-3, the bactericidal activity of the bromides also increased with an increase

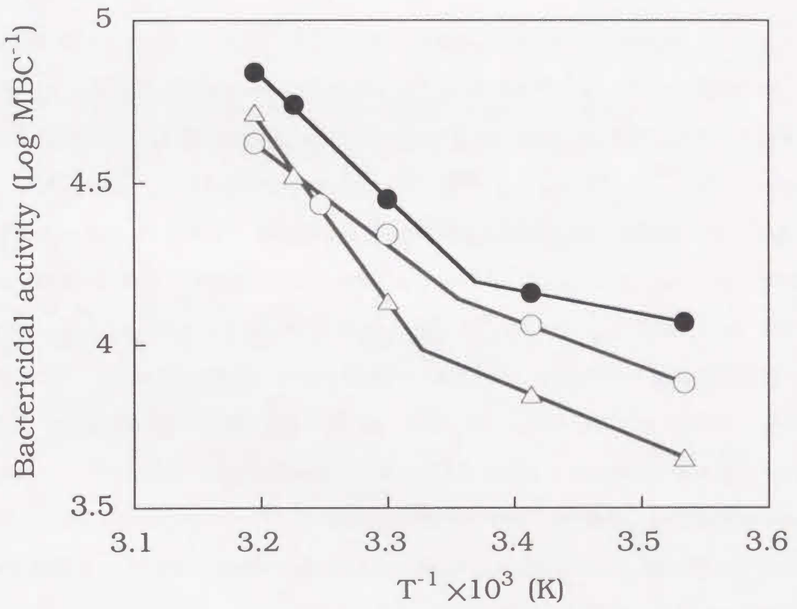


Fig. 3-3. Effect of temperature on the bactericidal activity (log MBC^{-1}) of ATP-12 (●), BNP-12 (○) and PBr-12 (△) against *E. coli* K12 W3110. MBCs were measured at 10, 20, 30, 35, 37 and 40℃.

in temperature, and each plot of log MBC^{-1} against the reciprocal of the absolute temperature gave two straight lines. It is thought that the temperature of the intersection of two lines is a phase transition temperature of the bacterial cell membrane. The fluidity of cell membrane is closely associated with the phase transition temperature, that is, the membrane becomes more fluid over that temperature. Therefore, it seems that the mode of bactericidal action of the bromides is closely related to the fluidity of the bacterial cell membrane.

3-3-5. *Bacterioclastic activity*

Critical vesiculation concentrations (CVCs) against exponential-phase cells of *E. coli* K12 W3110 were measured in terms of the increase in turbidity of the cell suspensions (Kourai et al., 1994a). The bacterioclastic activity (log CVC^{-1}) means the ability of the QACs to destroy the structure of bacterial cell surfaces. Figure 3-4 shows the bacterioclastic activity of ATP-n, BNP-n and PBr-n. The activity of ATP-n and BNP-n, except for the bromides with decyl group, were approximately constant, and the activity was scarcely influenced by alkyl chain length, that is, the molecular hydrophobicity. The bacterioclastic activity of all ATP-n was much higher than that of the corresponding BNP-n. From this result, it is implied that the introduction of an allylthio group on the pyridine ring enhances the bacterioclastic activity of pyridinium derivatives.

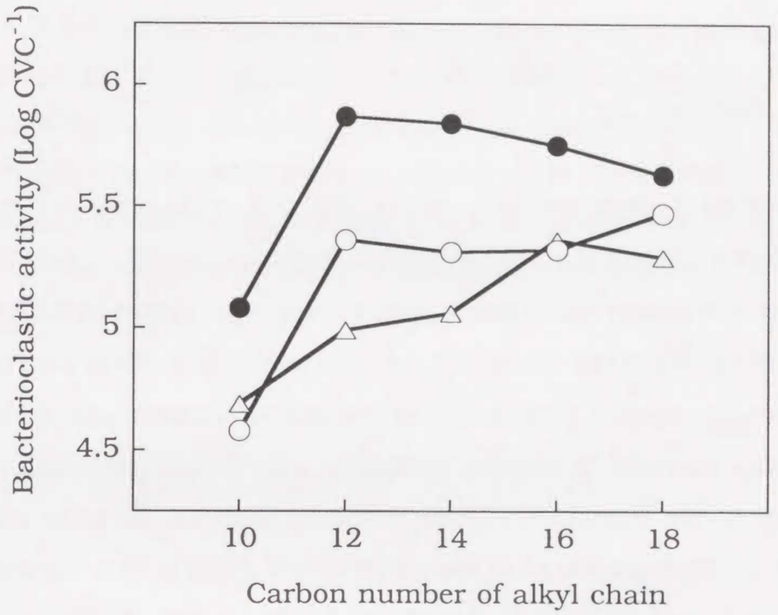


FIG. 3-4. Comparison of bacterioclastic activity (log CVC^{-1}) of ATP-n (●), BNP-n (○) and PBr-n (△) against *E. coli* K12 W3110.

Chapter 4. Antibacterial Characteristics of *N*-Alkyl-2-alkylthiopyridinium and *N*-Alkyl-4-alkylthiopyridinium Salts

4-1. Summary

N-Alkyl-2-alkylthiopyridinium (2TPX-n: X=I or Br, n=6−18) and *N*-alkyl-4-alkylthiopyridinium salts (4TPX-n), which have an electron-releasing group on the pyridine ring, were synthesized. Both 2TPBr-12 and 4TPBr-12 showed wide and potent bactericidal spectra of activity against gram-negative bacteria (9 strains) and gram-positive bacteria (3 strains), compared with *N*-dodecylpyridinium iodide (P-12). The activity of these new compounds was not correlated with the hydrophobicity of the bacterial cell surface. This suggests that the bactericidal mechanism of 2TPBr-12 and 4TPBr-12 is different from that of P-12. The bactericidal and bacteriostatic activity of the new compounds against *Escherichia coli* K12 W3110 was closely influenced by their alkyl chain length. Since they have two hydrophobic alkyl chains in their structure, it seems that hydrophobic association between the molecule of 2TPX-n or 4TPX-n and medium components in the MIC measurement system caused the reduction in their apparent concentration. The bactericidal activity of these compounds was dependent on their bacterioclastic activity, and less dependent on their hydrophobicity (R_M).

4-2. Materials and Methods

4-2-1. *Chemicals*

2-Mercaptopyridine, 4-mercaptopyridine and *n*-alkylhalides (hexyliodide, octyliodide, octylbromide, decylbromide, dodecylbromide, tetradecylbromide, hexadecylbromide and octadecylbromide) were purchased from Kanto Chemical Co., Inc. Chemical structures of QACs used in this study are shown in Fig. 4-1. 2TPX-n and 4TPX-n were synthesized as described below.

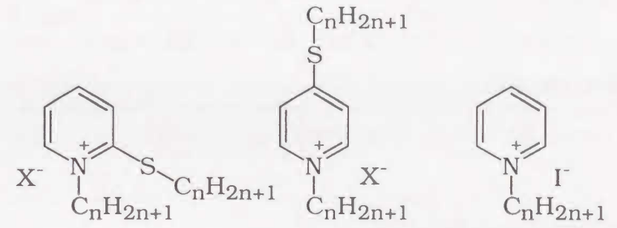


Fig. 4-1. Chemical structures of *N*-alkyl-2-alkylthiopyridinium salts (2TPX-n), *N*-alkyl-4-alkylthiopyridinium salts (4TPX-n) and *N*-alkylpyridinium iodides (P-n). The abbreviations, X and n, indicate the counter ion (Br^- or I^-) and the carbon numbers of alkyl chains, respectively.

4-2-2. *Synthesis of N-alkyl-2-alkylthiopyridinium salts*

A solution of 2-mercaptopyridine (0.05 mol) and alkylhalide (0.05 mol) in ethyl alcohol (100 ml) was refluxed for 3 h. The solvent was

removed by evaporation, and the residue was washed with diethyl ether (200 ml). Recrystallization from ethyl acetate gave 2-alkylthiopyridine hydrohalide. This product was made strongly basic to litmus with 1 N aqueous NaOH and extracted with diethyl ether. Evaporation of the ethyl ether gave 2-alkylthiopyridine. A mixture of 2-alkylthiopyridine (0.02 mol), alkylhalide (0.02 mol) and ethyl alcohol (50 ml) was heated at 80℃ for 72 h under 80 MPa of static pressure with a YHP-92 high pressure reactor. Solvent was removed by evaporation, then the residue was recrystallized twice from ethyl acetate to give the title compound.

4-2-3. *Synthesis of N-alkyl-4-alkylthiopyridinium salts*

N-Alkyl-4-alkylthiopyridinium salts were prepared by procedures similar to those for the *N*-alkyl-2-alkylthiopyridinium salts.

4-2-4. *Hydrophobicity of cell surface*

The hydrophobicity of the cell surface of *M. luteus* was determined using the partition system of *n*-hexadecane-physiological saline, as described in Chapter 2.

4-2-5. *Scanning electron microscopy*

The exponential-phase cell suspension of *E. coli* K12 was treated using a 2TPBr-12 (20.4 μM) at 37℃ for 200 s. The following procedure is described in Chapter 1.

4-3. Results and Discussion

4-3-1. *Chemical properties of 2TPX-n and 4TPX-n*

Table 4-1 shows the analytical data and yields of the synthesized compounds. The hexyliodide and octyliodide were used for the synthesis of 2TPI-6, 4TPI-6 and 4TPI-8 to raise their total yields. The results of the elemental analysis are in fair agreement with their theoretical values. The NMR data (δ) are consistent with the proposed structures of the compounds. The following data on 4TPI-8 is given as a typical result. The signals of 3.29 (t, 2H, *J*=7.1 Hz) and 4.50 (t, 2H,

Table 4-1. Chemical properties and yields of synthesized *N*-alkyl-2-alkylthiopyridinium salts (2TPX-n) and *N*-alkyl-4-alkylthiopyridinium salts (4TPX-n)

Compound (abbrev.)	Elemental analysis (%)						m.p. (°C)	Yield (%)
	C		H		N			
	Found	Calc.	Found	Calc.	Found	Calc.		
2TPI-6	49.87	50.12	7.23	7.42	3.30	3.44	92–93	61.8
2TPBr-8	60.40	60.56	9.05	9.20	3.13	3.36	114–117	6.7
2TPBr-10	63.41	63.53	9.60	9.81	2.69	2.96	109–111	7.6
2TPBr-12	65.59	65.88	10.34	10.29	2.86	2.65	105–107	6.1
2TPBr-14	67.50	67.78	10.80	10.69	2.30	2.40	108–110	6.0
2TPBr-16	69.13	69.34	11.23	11.01	2.14	2.19	109–112	6.3
2TPBr-18	70.43	70.65	11.40	11.28	1.90	2.01	112–113	7.2
4TPI-6	49.85	50.12	7.16	7.42	3.56	3.44	41–43	63.7
4TPI-8	54.18	54.41	8.06	8.26	2.92	3.02	68–70	77.6
4TPBr-10	63.24	63.53	9.58	9.81	3.00	2.96	77–80	80.8
4TPBr-12	65.58	65.88	10.21	10.29	2.56	2.65	91–94	68.5
4TPBr-14	67.52	67.78	10.39	10.69	2.33	2.40	88–90	69.0
4TPBr-16	69.05	69.34	10.83	11.01	2.19	2.19	101–104	75.5
4TPBr-18	70.60	70.65	11.14	11.28	1.98	2.01	87–90	73.3

J=7.6 Hz) were assigned to the methylene protons of S-CH_2- and N^+-CH_2-, respectively. The signals of 7.87 (d, 2H, J=6.3 Hz) and 8.67 (d, 2H, J=6.8 Hz) were assigned to the aromatic protons of the *p*-substituted pyridine ring. The signal of 0.89 (t, 6H, J=6.1 Hz) was assigned to the terminal methyl protons of -CH_3. The NMR data on the other compounds are also consistent with their structures (data not shown).

4-3-2. *Antibacterial activity*

Table 4-2 shows the MBCs of 2TPBr-12 and 4TPBr-12 against gram-negative bacteria (9 strains) and gram-positive bacteria (3 strains). In order to investigate the effect of the introduction of an alkylthio group on the pyridine ring on the bactericidal action, the MBCs of P-12, one of the typical QACs with no substituents on the pyridine ring, were compared with those of 2TPBr-12 and 4TPBr-12. The new compounds had broad bactericidal spectra and higher bactericidal activity, expressed as log MBC^{-1}, against all the tested bacteria except for S. *aureus*. Especially against gram-negative bacteria, they showed much higher bactericidal activity than P-12. This result shows that the introduction of an alkylthio group enhances the bactericidal activity of the *N*-alkylpyridinium compounds.

In addition, the bactericidal activity of QACs tends to depend on the hydrophobicity of the bacterial cell surfaces (Kourai et al., 1989). The hydrophobicity of the bacteria used in this study are also listed in

Table 4-2. Minimum bactericidal concentrations (MBCs) of 2TPBr-12, 4TPBr-12 and P-12 against exponential-phase cells of gram-negative bacteria and gram-positive bacteria and hydrophobicity (log *HI*) of the bacterial cell surface.

Strain	MBC(μM)			log *HI*
	2TPBr-12	4TPBr-12	P-12	
Pseudomonas aeruginosa ATCC 10145	20	20	640	-1.21
Pseudomonas aeruginosa IFO 3080	12	20	327	-1.41
Klebsiella pneumoniae ATCC 4352	15	10	85	-0.81
Klebsiella pneumoniae ATCC 13883	15	16	205	-1.10
Proteus rettgeri NIH 96	20	5	200	-0.81
Proteus mirabilis IFO 3849	32	25	250	-0.61
Escherichia coli IFO 3301	32	20	83	-0.68
Escherichia coli K12 OUT 8401	32	12	65	-0.79
Escherichia coli K12 W3110	20	60	83	-0.80
Bacillus subtilis IFO 3134	5	3	13	-0.72
Staphylococcus aureus ATCC 25923	14	16	12	-0.37
Micrococcus luteus IFO 12708	16	10	16	-0.35

Table 4-2. A higher value of log *HI* means a higher hydrophobicity. Generally, QACs are more effective against bacteria having hydrophobic cell surfaces, such as *S. aureus* and *M. luteus*, than against bacteria having hydrophilic cell surfaces, such as *Ps. aeruginosa* and *K. pneumoniae*. As seen in Fig. 4-2, the bactericidal activity of P-12 was dependent on the hydrophobicity of the cell surface. This indicates that P-12 interacts with the hydrophobic site of the bacterial cell surface. It is suggested that the cationic moiety of P-12 adsorbs to anionic sites on the bacterial cell surface, such as phosphate residues, and then the hydrophobic interaction between the alkyl group of P-12 and the hydrophobic sites on the bacterial cell surface occurs. Also, there are no correlations between 2TPBr-12 or 4TPBr-12 and the hydrophobicity, so that the bactericidal activity of the new compounds is not dependent on the hydrophobicity. This result suggests that the bactericidal mechanism of 2TPBr-12 and 4TPBr-12 is different from that of P-12.

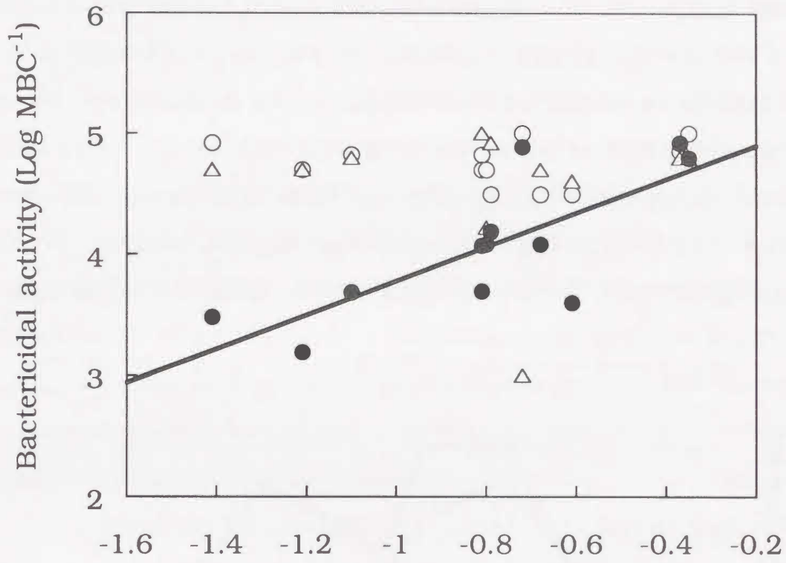


Fig. 4-2. Relationship between the bactericidal activity (log MBC^{-1}) of 2TPBr-12 (○), 4TPBr-12 (△) or P-12 (●) and hydrophobicity of exponential-phase cell surfaces. The hydrophobicity was determined using the partition system *n*-hexadecane-physiological saline. See text for details.

4-3-3. *Effect of alkyl chain length on antibacterial activity*

Figure 4-3 shows the influence of the alkyl chain length of 2TPX-n, 4TPX-n and P-n on their activity. The bacteriostatic activity of all tested compounds was significantly influenced by the length of the alkyl chain attached to the ammonium nitrogen. In the case of P-n, the activity increased with the length of the chain of the *N*-alkyl substituent and reached a maximum with C14. On the other hand, the new compounds having relatively short alkyl groups (2TPBr-8, 4TPI-8 and 4TPBr-10) exhibited the highest activity in the homologous series. There are no data of the salts having hexadecyl

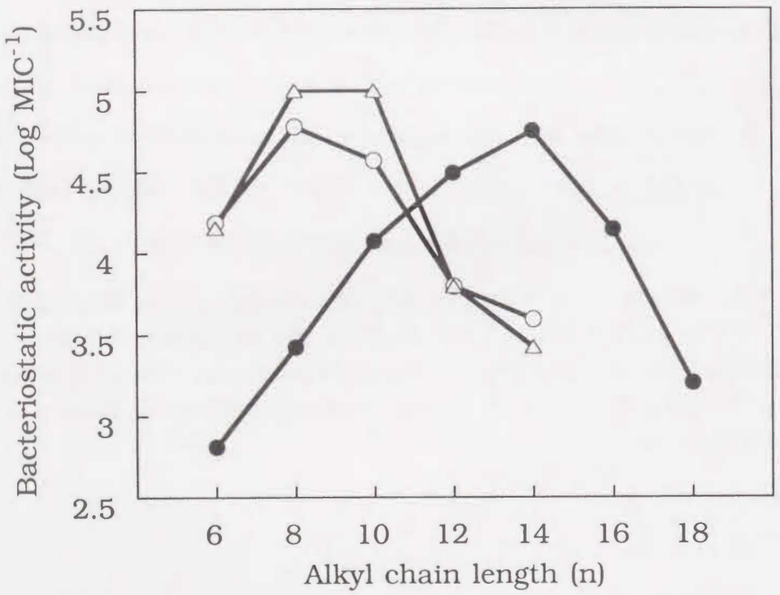


Fig. 4-3. Effect of alkyl chain length of 2TPX-n (○), 4TPX-n (△) and P-n (●) on bacteriostatic activity (log MIC^{-1}) against *E. coli* K12 W3110.

and octadecyl groups because of their insolubility in nutrient broth. The activity of the salts was not influenced by the nature of their counter ions (data not shown). The compounds having longer alkyl groups than the above compounds (2TPBr-8, 4TPI-8 and 4TPBr-10) exhibited much lower activity. One reason for this may be that there is an interaction between the compounds and medium components in the MIC measurement system. Therefore the influence of peptone on the activity of 4TPBr-12 was investigated using the MBC measurement system. As expected, MBCs of 4TPBr-12 increased with an increase in the peptone concentration (Table 4-3). The MBC was 1,000 μM with 0.5 % (w/v) peptone, which equals the concentration in nutrient broth. Since 2TPX-n and 4TPX-n have two hydrophobic alkyl chains, the interaction between the compounds and peptone must be much greater than that of P-n. It is thought that the hydrophobic association described above caused the reduction in the apparent concentration of the compounds, especially those having long alkyl chains, in the MIC measurement system, and this resulted in a reduction in the bacteriostatic activity.

Table 4-3. Effect of peptone on the minimum bactericidal concentration (MBC) of 4TPBr-12 against *E. coli* K12.

	Peptone (%, w/v)				
	0	0.05	0.1	0.2	0.5
MBC[a)] (μM)	60	328	512	800	1000

a) MBC of 4TPBr-12 was determined in the presence of peptone.

In the MBC measurement system, interactions between the compounds and medium components are negligibly small, since the

compounds were dissolved in sterile water and the cells were also suspended in sterilized water. After the compounds were incubated with the cell suspensions for 30 min at 37℃, a small amount of the mixture (0.1 ml) was taken out and added to the medium. As seen in Fig. 4-4, the alkyl chain length of P-n had a significant influence on the bactericidal activity. In contrast, in case of 2TPX-n and 4TPX-n, the influence of the alkyl chain length was relatively small and the activity remained almost constant from C6 to C14. From these results, it may be concluded that the antibacterial activity of 2TPX-n and 4TPX-n is markedly lowered by the presence of other material such as peptone in the measurement system, since they have two hydrophobic alkyl groups.

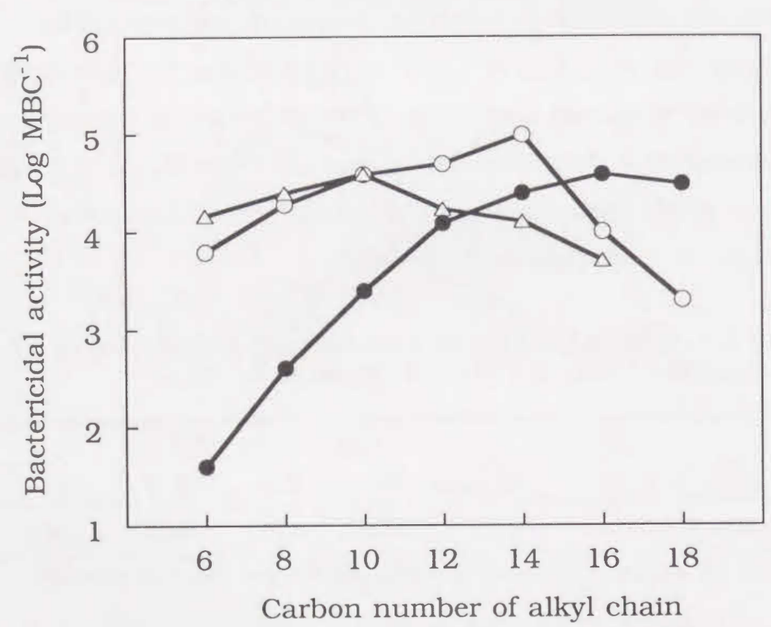


Fig. 4-4. Effect of alkyl chain length of 2TPX-n (○), 4TPX-n (△) and P-n (●) on bactericidal activity (log MBC^{-1}) against exponential-phase cells of *E. coli* K12 W3110.

4-3-4. *Turbidity in the cell suspension*

The optical density (OD_{660}) of the cell suspension of *E. coli* K12 W3110 containing 2TPBr-12 was continuously followed for 200 s. As seen in Fig. 4-5A, 2TPBr-12 as well as other pyridinium derivatives used in this study caused an increase in the turbidity of the cell suspension. The turbidity reached a maximum during the first 60-120 s and the extent was related to the 2TPBr-12 concentration. The increase (ΔOD_{660}/200 s) in turbidity was plotted against the concentration (0.83−16.7 μM) of 2TPBr-12 giving two straight lines as shown in Fig. 4-5B. The CVC calculated from two regression equations is 6.1 μM.

4-3-5. *Electron microscopy*

To investigate the phenomenon of increased turbidity caused by the QACs, the exponential-phase cells of *E. coli* K12 treated with 2TPBr-12 for 200 s was monitored by scanning electron microscopy. The concentration of 2TPBr-12 required was equimolar with the MBC (20.4 μM). As seen in Fig. 4-6, the treated cells were injured and formed many blebs on the cell surfaces. In the case of untreated cells, no bleb was observed (figure not shown). This shows that the formation of these blebs was induced by 2TPBr-12. It is suggested that 2TPBr-12 interacts with hydrohobic sites on the bacterial cell surface, and turbid materials are formed from the outer membrane and/or cytoplasmic membrane and/or intracellular materials, after destruction of the cell structure.

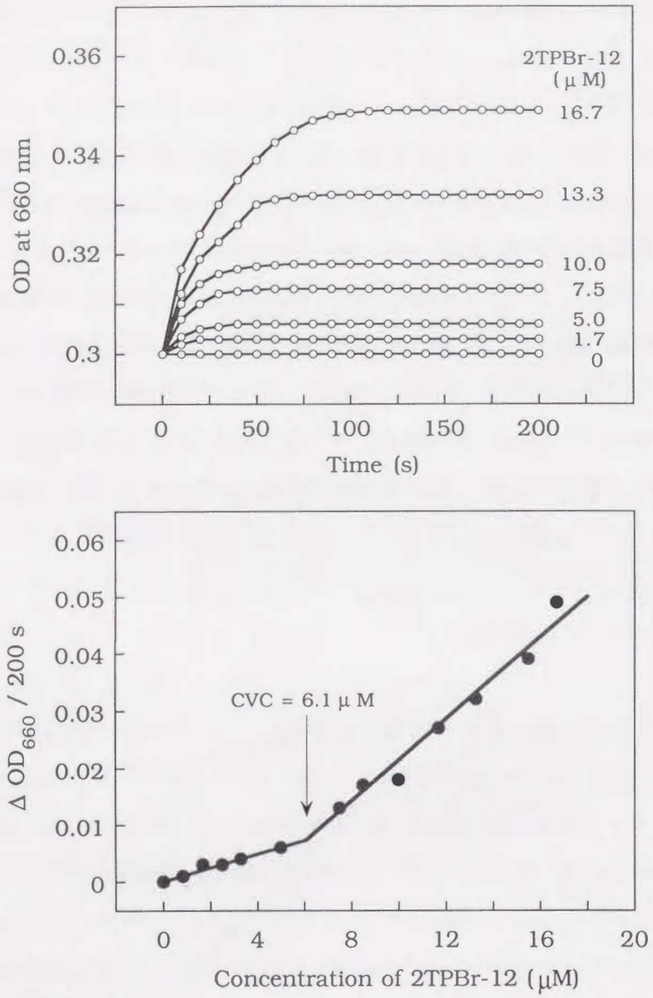


Fig. 4-5. (A) Turbidity increase of the exponential-phase cell suspensions of *E. coli* K12 W3110 treated with 2TPBr-12. The turbidity was measured at 37℃. The numerical values indicate the concentrations of 2TPBr-12.
(B) Effect of the concentration of added 2TPBr-12 on the turbidity increase (ΔOD at 660 nm for 200 s) of the cell suspensions. The arrow indicates the critical vesiculation concentration (CVC).

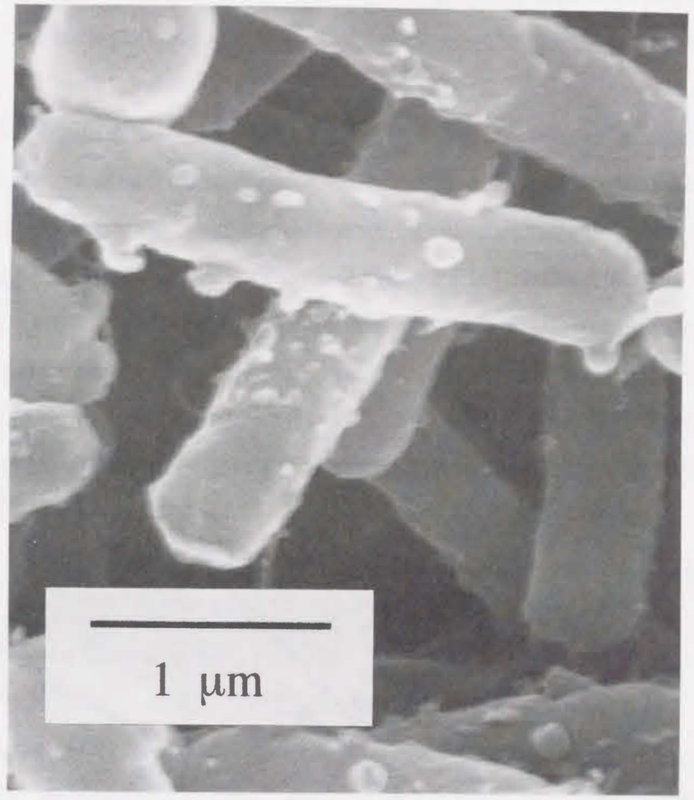


Fig. 4-6. Scanning electron micrograph of *E. coli* K12 W3110 treated with 20.4 μM of 2TPBr-12 for 200 s.

4-3-6. *Relationship between bactericidal activity and bacterioclastic activity*

The results described above suggest that the phenomenon of increased turbidity is closely related to the bactericidal activity of the compounds, as previously reported by Kourai et al. (1995). The bacterioclastic activity (log CVC^{-1}) describes the ability of QACs to destroy the structure of bacterial cell surfaces. Figure 4-7 shows the relationship between the bactericidal activity and the bacterioclastic activity of 2TPX-n, 4TPX-n and P-n. The log MBC^{-1} of each QAC was observed to increase linearly with log CVC^{-1}. This suggests that the

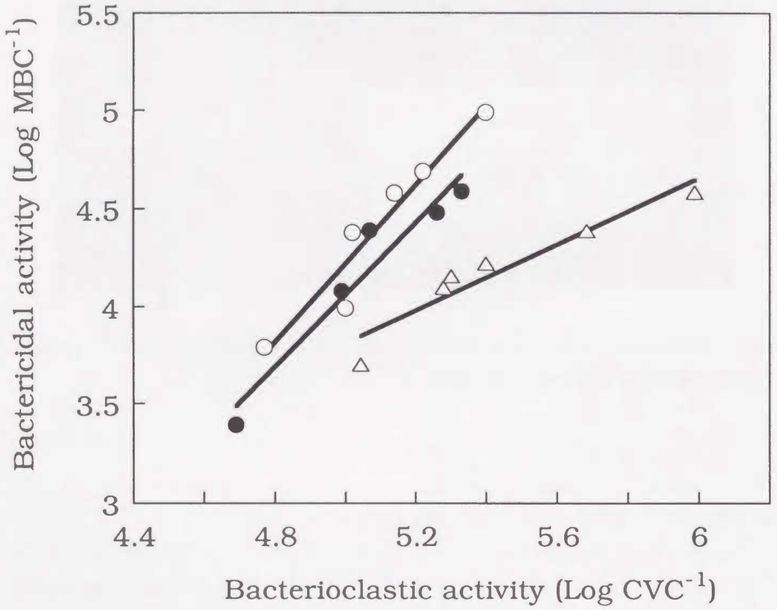


Fig. 4-7. Relationship between bactericidal activity (log MBC^{-1}) and bacterioclastic activity (log CVC^{-1}) of 2TPX-n (○), 4TPX-n (△) and P-n (●) against *E. coli* K12 W3110.

bactericidal activity of these QACs against *E. coli* K12 is dependent on their bacterioclastic activity, and *vice versa*. The dependence of 4TPX-n, however, was smaller than that of 2TPX-n and P-n. It is thought that the molecular size of 4TPX-n in aqueous solution is larger than that of the others, because they are *p*-substituted pyridinium compounds. The bactericidal mechanism of 4TPX-n, such as the interaction at the target site, may be influenced by the bulk of the molecule, and the action may be slightly dependent on the bacterioclastic activity.

4-3-7. *Relationship between bactericidal activity and hydrophobicity*

The bactericidal activity of the compounds used in this study against *E. coli* K12 was plotted against their hydrophobicity (R_M). The curves were found to be parabolic (Fig. 4-8). The activity of P-n was very dependent on the hydrophobicity, while that of 2TPX-n and 4TPX-n was not. This and the result shown in Table 4-2 suggest that the alkylthio group on the pyridine ring acts on the bacterial cell surfaces producing a small hydrophobic effect.

4-3-8. *Effect of temperature on bactericidal activity*

The MBCs of 2TPBr-12, 4TPBr-12 and P-12 against the exponential-phase cells of *E. coli* K12 were measured at 10, 20, 30, 37, and 40℃ in order to investigate the interaction between the bacterial cell surfaces and the study compounds. In general, increasing temperature tends to increase the bactericidal activity of QACs. The bactericidal activity of the salts used in this study also increased with

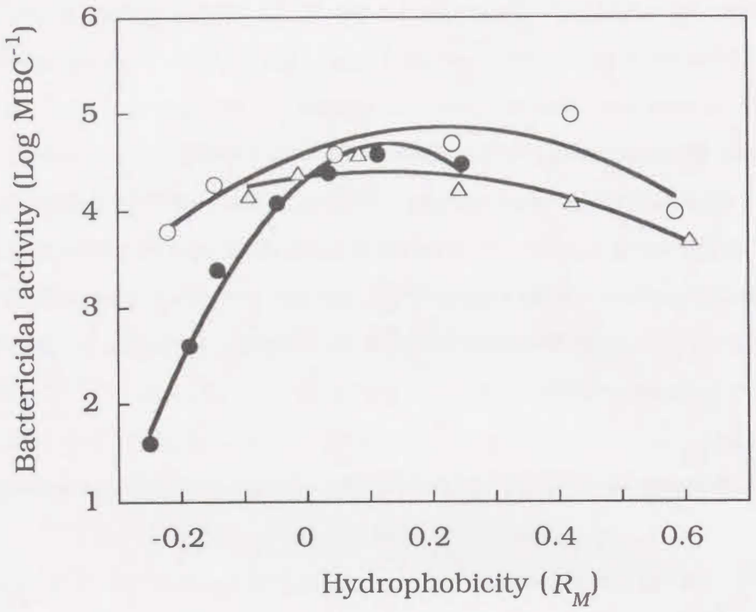


Fig. 4-8. Relationship between bactericidal activity (log MBC^{-1}) and hydrophobicity (R_M) of 2TPX-n (○), 4TPX-n (△) and P-n (●) against *E. coli* K12 W3110.

an increase in temperature, and each plot of log MBC^{-1} against the reciprocal of the absolute temperature (T^{-1}) gave two straight lines (Fig. 4-9). The temperature at the intersection of the two lines seems to be the phase transition temperature of the bacterial cell membrane, and it is associated with the fluidity of the bacterial membrane. The temperatures at the intersection of 2TPBr-12 and 4TPBr-12 were lower than that of P-12. This suggests that the bacterial membrane treated with the new compounds brought about a phase transition at a lower temperature. As the mode of bactericidal action of QACs, which interact with the bacterial membrane, is closely related to the membrane fluidity, the difference in the

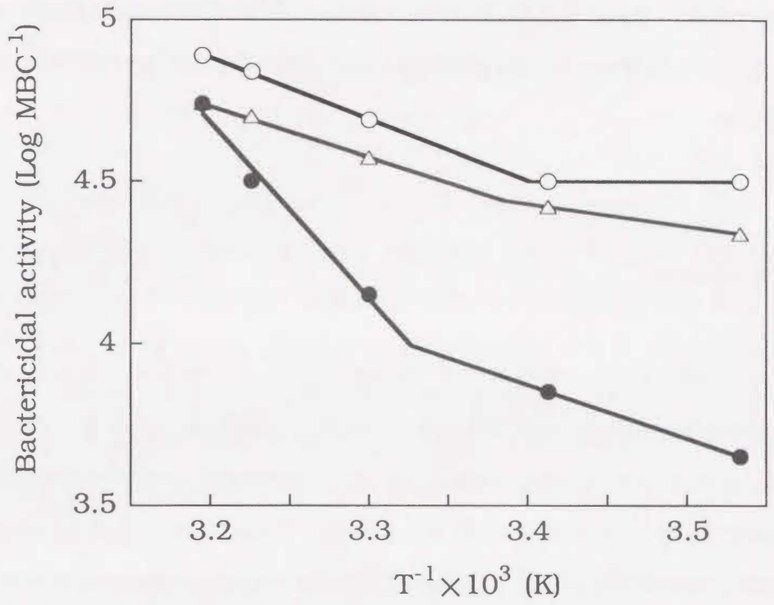


Fig. 4-9. Temperature-dependence of bactericidal activity (log MBC^{-1}) of 2TPBr-12 (○), 4TPBr-12 (△) and P-12 (●) against *E. coli* K12 W3110. The MBCs were measured at 10, 20, 30, 37, and 40℃.

temperatures implies that the new compounds and P-12 have different bactericidal mechanisms. In addition, the slopes of the lines for 2TPBr-12 and 4TPBr-12 were smaller than that for P-12. As bactericidal activity seems to be proportional to the bactericidal rate, the slope is regarded as an apparent activating energy of the bactericidal action of the salt. Therefore, it is thought that the activating energy of 2TPBr-12 and 4TPBr-12 is much smaller than that of P-12.

Chapter 5. Synthesis and Antimicrobial Characteristics of 4,4'-(α,ω-Polymethylenedithio)bis(1-alkylpyridinium iodide)s

5-1. Summary

Bis-quaternary ammonium compounds (bis-QACs), 4,4'-(α,ω-polymethylenedithio)bis(1-alkylpyridinium iodide)s (4DTBP-m,n), which have 3 to 10 carbon atoms in the connecting methylene chain (m) and 8 to 18 carbon atoms of the *N*-alkyl chain (n), were synthesized. 4DTBP-6,12 exhibited a wide antimicrobial spectrum against gram-positive and gram-negative bacteria and fungi. The activity was stronger than those of *N*-dodecylpyridinium iodide (P-12), benzyldodecyldimethylammonium chloride and 2-(4-thiazolyl)benzimidazole. The bactericidal activities of 4DTBP-m,n were scarcely affected by the lengths of the alkyl chain and methylene chain. The bis-QAC that showed the highest activity was 4DTBP-6,8 (MIC = 1.6 μM, MBC = 2.6 μM), and its activity was about 10 times that of *N*-hexadecylpyridinium iodide (P-16), which was the most active in the P-n series. In addition, 4DTBP-6,12 showed a high bactericidal activity in the ranges of pH 5 to 8.5 and 10 to 40℃, in contrast to mono-QACs. The bis-QACs synthesized in this study have excellent bactericidal properties.

5-2. Materials and Methods

5-2-1. *Chemicals*

The bis-QACs listed in Table 5-1 were synthesized from the corresponding polymethylene bromide (trimethylene bromide, tetramethylene bromide, hexamethylene bromide, octamethylene bromide and decamethylene bromide), *n*-alkyl iodide (hexyl iodide, octyl iodide, decyl iodide, dodecyl iodide, tetradecyl iodide, hexadecyl iodide and octadecyl iodide) and 4-mercaptopyridine (Chart 5-1). Benzyldodecyldimethylammonium chloride (10 % (w/v) benzalkonium chloride solution; BAC) was purchased from Takeda Pharmaceutical Co., Ltd. (Osaka) and 2-(4-thiazolyl)benzimidazole (TBZ) was obtained from San-ai oil Co., Ltd. (Tokyo) (Fig. 5-1).

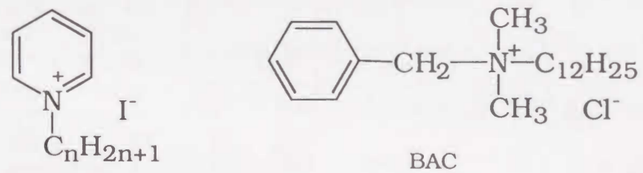
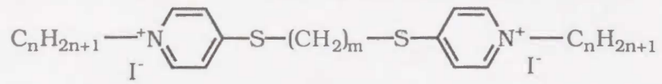


Fig. 5-1. Chemical structures of *N*-alkylpyridinium iodide (P-n), benzyldodecyldimethylammonium chloride (BAC) and 2-(4-thiazolyl)benzimidazole (TBZ).

Table 5-1. Physical properties of 4,4'-(α, ω-polymethylenedithio)bis(1-alkylpyridinium iodide)s (4DTBP-m,n).



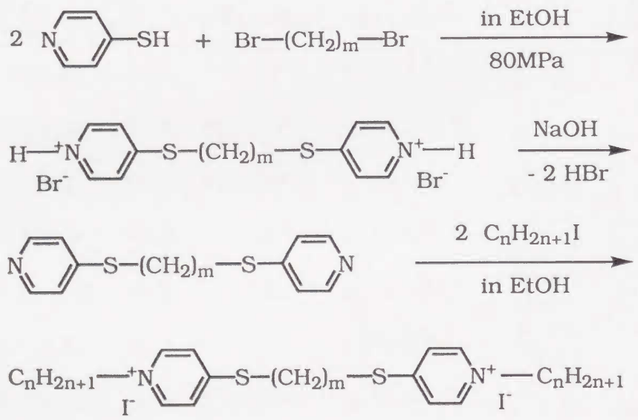
Compd.	Yield (%)	m.p. (℃)	Formula	Elemental analysis (%) Calcd. (found)		
				C	H	N
4DTBP-3,8	80.5	125-128	$C_{29}H_{48}I_2N_2S_2$	46.90 (46.81	6.51 6.25	3.77 3.51)
4DTBP-3,10	81.2	159-161	$C_{33}H_{56}I_2N_2S_2$	49.62 (49.43	7.07 6.94	3.51 3.78)
4DTBP-3,12	80.3	169-170	$C_{37}H_{64}I_2N_2S_2$	51.99 (51.87	7.55 7.29	3.28 2.99)
4DTBP-3,14	82.1	174-175	$C_{41}H_{72}I_2N_2S_2$	54.06 (53.78	7.97 7.77	3.08 2.93)
4DTBP-3,16	83.4	166-169	$C_{45}H_{80}I_2N_2S_2$	55.89 (55.80	8.34 8.04	2.90 2.98)
4DTBP-3,18	85.0	149-151	$C_{49}H_{88}I_2N_2S_2$	57.52 (57.25	8.67 8.33	2.74 2.71)
4DTBP-4,8	80.0	147-149	$C_{30}H_{50}I_2N_2S_2$	47.62 (47.37	6.66 6.53	3.70 4.00)
4DTBP-4,10	81.2	149-151	$C_{34}H_{58}I_2N_2S_2$	50.24 (49.95	7.19 6.96	3.45 3.40)
4DTBP-4,12	80.7	168-171	$C_{38}H_{66}I_2N_2S_2$	52.53 (52.34	7.66 7.38	3.22 3.01)
4DTBP-4,14	80.5	165-168	$C_{42}H_{74}I_2N_2S_2$	54.54 (54.31	8.06 8.00	3.03 3.02)
4DTBP-4,16	83.2	175-178	$C_{46}H_{82}I_2N_2S_2$	56.31 (56.08	8.42 8.21	2.86 3.12)
4DTBP-4,18	84.2	165-168	$C_{50}H_{90}I_2N_2S_2$	57.90 (57.65	8.75 8.50	2.70 2.66)
4DTBP-6,8	88.0	102-103	$C_{32}H_{54}I_2N_2S_2$	48.98 (48.93	6.94 6.80	3.57 3.57)
4DTBP-6,10	87.2	121-122	$C_{36}H_{62}I_2N_2S_2$	51.42 (51.54	7.43 7.28	3.33 3.39)
4DTBP-6,12	87.3	136-137	$C_{40}H_{70}I_2N_2S_2$	53.56 (53.47	7.87 7.63	3.12 3.09)
4DTBP-6,14	86.5	135-136	$C_{44}H_{78}I_2N_2S_2$	55.45 (55.15	8.25 7.96	2.94 2.74)
4DTBP-6,16	86.8	140-141	$C_{48}H_{86}I_2N_2S_2$	57.13 (57.06	8.59 8.41	2.78 2.77)
4DTBP-6,18	87.0	138-139	$C_{52}H_{94}I_2N_2S_2$	58.63 (58.38	8.89 8.97	2.63 2.40)

Table 5-1. (continued)

Compd.	Yield (%)	m.p. (℃)	Formula	Elemental analysis (%) Calcd. (found)		
				C	H	N
4DTBP-8,8	84.3	87-89	$C_{34}H_{58}I_2N_2S_2$	50.24 (49.96	7.19 7.07	3.45 3.74)
4DTBP-8,10	83.2	122-123	$C_{38}H_{66}I_2N_2S_2$	52.53 (52.32	7.66 7.57	3.22 3.09)
4DTBP-8,12	86.5	132-133	$C_{42}H_{74}I_2N_2S_2$	54.54 (54.31	8.06 7.85	3.03 2.73)
4DTBP-8,14	84.6	137-138	$C_{46}H_{82}I_2N_2S_2$	56.31 (56.01	8.42 8.27	2.86 3.11)
4DTBP-8,16	83.5	148-149	$C_{50}H_{90}I_2N_2S_2$	57.90 (57.68	8.75 8.55	2.70 2.65)
4DTBP-8,18	84.0	155-157	$C_{54}H_{98}I_2N_2S_2$	59.32 (59.15	9.03 8.73	2.56 2.49)
4DTBP-10,8	82.1	119-120	$C_{36}H_{62}I_2N_2S_2$	51.42 (51.12	7.43 7.17	3.33 3.18)
4DTBP-10,10	82.6	128-129	$C_{40}H_{70}I_2N_2S_2$	53.56 (53.43	7.87 7.84	3.12 3.36)
4DTBP-10,12	84.6	134-136	$C_{44}H_{78}I_2N_2S_2$	55.45 (55.15	8.25 8.10	2.94 3.22)
4DTBP-10,14	85.3	142-144	$C_{48}H_{86}I_2N_2S_2$	57.13 (56.86	8.59 8.33	2.78 2.56)
4DTBP-10,16	85.6	148-149	$C_{52}H_{94}I_2N_2S_2$	58.63 (58.65	8.89 8.65	2.63 2.54)
4DTBP-10,18	85.6	152-154	$C_{56}H_{102}I_2N_2S_2$	60.00 (59.76	9.17 8.95	2.50 2.39)

5-2-2. *Synthesis of 4,4'-(α,ω-polymethylenedithio)bis(1-alkylpyridinium iodide)s*

A solution of 4-mercaptopyridine (1.0 mol) and polymethylene bromide (0.5 mol) in ethyl alcohol was refluxed for 4 h. The solvent was removed by evaporation, and the residue was recrystallized from ethyl alcohol-water (9:1) to give 4,4'-(α,ω-polymethylenedithio) bis-pyridine hydrobromides. This product was made strongly basic to litmus with 0.1 N aqueous NaOH and extracted with hot benzene (50℃). Evaporation of the benzene gave 4,4'-(α,ω-polymethylene-dithio)bispyridine. A mixture of the bispyridine (0.1 mol), *n*-alkyl iodide (0.2 mol) and ethyl alcohol (100 ml) was refluxed for 48 h. The solvent was removed by evaporation, and the residue was recrystallized twice from ethyl acetate-ethyl alcohol to give the title compounds.



4DTBP-m,n m=3,4,6,8,10
n=8,10,12,14,16,18

Chart 5-1.

5-2-3. *Minimum inhibitory concentration against molds*

The MICs against molds were measured by a broth dilution method. A Sabouraud broth (Nissui Pharmaceutical Co., Ltd., Tokyo) was used for the antifungal test. Molds were incubated on Sabouraud-agar plates for 7 d at 28℃. Spore suspensions were prepared by adding sterile physiological saline (10 ml) containing 0.2 % (v/v) Tween-80 to the plates. The spore suspension was diluted 1000-fold with Sabouraud broth. Two-ml portions of the diluted spore suspension were mixed with 2 ml portions of diluted QACs and incubated at 20℃ for 48 h. The MICs against molds were determined.

5-3. Results and Discussion

5-3-1. *Chemical properties of 4DTBP-m,n*

Analytical and physical data for 4DTBP-m,n are summarized in Table 5-1 and ^{1}H-NMR data for the series of 4DTBP-m,8 and 4DTBP-6,n are summarized in Table 5-2.

5-3-2. *Antimicrobial activity*

The new bis-QAC, 4DTBP-6,12, was tested for antimicrobial activity against gram-negative bacteria (10 strains), gram-positive bacteria (9 strains) and fungi (7 strains). A typical mono-QAC (P-12), which has the pyridinium moiety of 4DTBP-6,12, and BAC, which is a QAC commercially available as a disinfectant, were also measured for comparison. Table 5-3 shows their MICs against various bacteria. 4DTBP-6,12 exhibited a wide antibacterial spectrum and a strong bacteriostatic activity against all bacteria tested in this study. The activity was much higher than those of P-12 and BAC, especially against gram-negative bacteria. In general, mono-QACs such as P-12 and BAC are more effective against gram-positive bacteria than against gram-negative bacteria (Maeda et al., 1996). As the cell surfaces of gram-positive bacteria are more hydrophobic than those of gram-negative bacteria (Kourai et al., 1989), it is thought that gram-positive bacteria have a higher susceptibility to compounds which interact with the cell surfaces. Bis-QAC (4DTBP-6,12), however, showed high bacteriostatic activity against both gram-negative and gram-positive bacteria. Other bis-QACs synthesized in this study also exhibited wide antibacterial spectra against the bacteria listed in Table 5-3 (data not shown). This may imply that the activities of 4DTBP-m,n are independent of the hydrophobicity of the bacterial cell surface.

Table 5-4 shows the MICs against fungi of 4DTBP-6,12, P-12 and TBZ, which is one of the most widely used antifungal agents. Though QACs are generally not effective against fungi (Kourai et al., 1994), 4DTBP-6,12 exhibited a wide spectrum and a high antifungal activity in addition to its antibacterial activity (Table 5-2). The activity (MIC = 2.8-20.5 μM) was much higher than those of P-12 and TBZ.

Table 5-2. ^{1}H-NMR data for 4DTBP-m,8 and 4DTBP-6,n.

Compd.	^{1}H-NMR (CD_3OD) δ
4DTBP-3,8	0.88 (6H, t, *J*=6.8Hz), 1.29-1.38 (20H, m), 1.99 (4H, m), 2.27 (2H, m), 3.56 (4H, t, *J*=7.3Hz), 4.54 (4H, t, *J*=7.6Hz), 7.98 (4H, d, *J*=6.8Hz), 8.75 (4H, d, *J*=6.8Hz)
4DTBP-4,8	0.89 (6H, t, *J*=6.8Hz), 1.29-1.38 (20H, m), 1.97 (4H, m), 2.03 (4H, m), 3.40 (4H, t, *J*=7.2Hz), 4.51 (4H, t, *J*=7.6Hz), 7.94 (4H, d, *J*=7.3Hz), 8.69 (4H, d, *J*=6.8Hz)
4DTBP-6,8	0.89 (6H, t, *J*=6.8Hz), 1.29-1.38 (20H, m), 1.62 (4H, m), 1.83 (4H, m), 1.97 (4H, m), 3.32 (4H, t, *J*=7.3Hz), 4.50 (4H, t, *J*=7.6Hz), 7.90 (4H, d, *J*=6.8Hz), 8.68 (4H, d, *J*=7.3Hz)
4DTBP-8,8	0.89 (6H, t, *J*=6.8Hz), 1.30-1.38 (20H, m), 1.42 (4H, m), 1.55(4H, m), 1.81 (4H, m), 1.98 (4H, m), 3.29 (4H, t, *J*=7.3Hz), 4.49 (4H, t, *J*=7.6Hz), 7.88 (4H, d, *J*=7.3Hz), 8.66 (4H, d, *J*=7.3Hz)
4DTBP-10,8	0.89 (6H, t, *J*=7.1Hz), 1.30-1.38 (28H, m), 1.53(4H, m), 1.79 (4H, m), 1.97 (4H, m), 3.28 (4H, t, *J*=7.3Hz), 4.48 (4H, t, *J*=7.6Hz), 7.86 (4H, d, *J*=6.8Hz), 8.65 (4H, d, *J*=6.8Hz)
4DTBP-6,10	0.89 (6H, t, *J*=6.8Hz), 1.29-1.38 (28H, m), 1.62 (4H, m), 1.83 (4H, m), 1.97 (4H, m), 3.32 (4H, t, *J*=7.1Hz), 4.49 (4H, t, *J*=7.3Hz), 7.89 (4H, d, *J*=6.3Hz), 8.67 (4H, d, *J*=6.4Hz)
4DTBP-6,12	0.89 (6H, t, *J*=6.8Hz), 1.28-1.37 (36H, m), 1.61 (4H, m), 1.83 (4H, m), 1.96 (4H, m), 3.30 (4H, t, *J*=7.6Hz), 4.44 (4H, t, *J*=7.3Hz), 7.85 (4H, d, *J*=7.3Hz), 8.59 (4H, d, *J*=7.3Hz)
4DTBP-6,14	0.89 (6H, t, *J*=6.8Hz), 1.28-1.37 (44H, m), 1.61 (4H, m), 1.83 (4H, m), 1.95 (4H, m), 3.31 (4H, t, *J*=7.6Hz), 4.43 (4H, t, *J*=7.3Hz), 7.85 (4H, d, *J*=7.3Hz), 8.58 (4H, d, *J*=7.3Hz)
4DTBP-6,16	0.89 (6H, t, *J*=6.8Hz), 1.28-1.37 (52H, m), 1.60 (4H, m), 1.83 (4H, m), 1.95 (4H, m), 3.31 (4H, t, *J*=7.6Hz), 4.43 (4H, t, *J*=7.6Hz), 7.84 (4H, d, *J*=7.3Hz), 8.58 (4H, d, *J*=7.3Hz)
4DTBP-6,18	0.90 (6H, t, *J*=6.6Hz), 1.28-1.37 (60H, m), 1.61 (4H, m), 1.83 (4H, m), 1.96 (4H, m), 3.27 (4H, t, *J*=7.6Hz), 4.43 (4H, t, *J*=7.6Hz), 7.84 (4H, d, *J*=6.4Hz), 8.58 (4H, d, *J*=5.9Hz)

Table 5-3. Minimum inhibitory concentrations (MICs) of 4DTBP-6,12, P-12, and BAC against gram-negative and gram-positive bacteria.

Strain	MICs (μM)[a]		
	4DTBP-6,12[b]	P-12[c]	BAC[d]
Pseudomonas aeruginosa ATCC 27583	10.0	256	53.7
Pseudomonas aeruginosa ATCC 10145	8.0	164	53.7
Pseudomonas aeruginosa IFO 3080	10.0	205	67.1
Klebsiella pneumoniae ATCC 4352	4.1	21.0	10.5
Klebsiella pneumoniae ATCC 13883	3.3	51.2	64.0
Proteus rettgeri NIH 96	4.1	100	53.7
Proteus vulgaris ATCC 13315	1.7	32.8	16.4
Proteus mirabilis IFO 3849	8.0	500	205
Escherichia coli K12 OUT 8401	2.6	26.2	10.5
Escherichia coli K12 W3110	5.1	64.0	21.0
Bacillus subtilis IFO 3134	2.1	8.4	4.2
Bacillus subtilis ATCC 6633	2.1	16.4	6.6
Bacillus cereus IFO 3001	3.3	12.8	6.6
Bacillus megaterium IFO 3003	3.3	16.4	6.6
Micrococcus luteus IFO 12708	2.1	16.0	6.7
Micrococcus lysodeikticus NCTC 2665	1.1	2.7	2.8
Staphylococcus aureus IFO 12732	1.1	4.2	5.4
Staphylococcus epidermidis ATCC 12228	2.1	6.6	6.6
Staphylococcus aureus JC1 (MRSA)	1.3	50.0	13.1

a) MICs were measured by a broth dilution method using nutrient broth at 37℃ for 24 h.
b) 4,4'-(1,6-Hexamethylenedithio)bis(1-dodecylpyridinium iodide)
c) Benzyldodecyldimethylammonium chloride
d) Dodecylpyridinium iodide

Table 5-4. Minimum inhibitory concentrations (MICs) of 4DTBP-6,12, P-12, and TBZ against fungi.

Strain	MIC (μM)[a]		
	4DTBP-6,12[b]	P-12[c]	TBZ[d]
Aspergillus terreus IFO 6346	8.4	131	156
Penicillium funiculosum IFO 6345	3.4	15.6	9.7
Chaetomium globosum IFO 6347	2.8	27.5	311
Cladosporium cladosporioides IFO 6348	8.4	27.5	9.7
Aureobasidium pullulans IFO 6353	16.4	67.1	38.8
Gliocladium virens IFO 6355	8.4	83.9	156
Rhizopus stolonifer IFO 4781	20.5	256	311

a) MICs were measured by a broth dilution method using sabouraud broth at 28 ℃ for 7 days.
b) 4,4'-(1,6-Hexamethylenedithio)bis(1-dodecylpyridinium iodide)
c) Dodecylpyridinium iodide
d) 2-(4-Thiazolyl)benzimidazole

5-3-3. *Effect of alkyl chain length on antibacterial activity*

The antibacterial activities of QACs are generally influenced by the length of the alkyl group attached to the pyridinium nitrogen atom (Kourai et al., 1980a). As can be seen in Fig. 5-2, the bacteriostatic activity of P-n was also affected by the length of the alkyl chain and was maximum with C16. In the case of 4DTBP-m,n, their activities increased when the molecules had shorter alkyl chains. On the other hand, the length of the chain connecting the two symmetrical quaternary ammonium moieties scarcely affected the activities. As the QACs tested in this study contain hydrophobic alkyl chains, it is plausible that there is a hydrophobic interaction between the molecule and medium components in the MIC measurement system. The bacteriostatic activities of QACs with long alkyl chains, such as P-18, could be inhibited by such an interaction. In the case of bis-

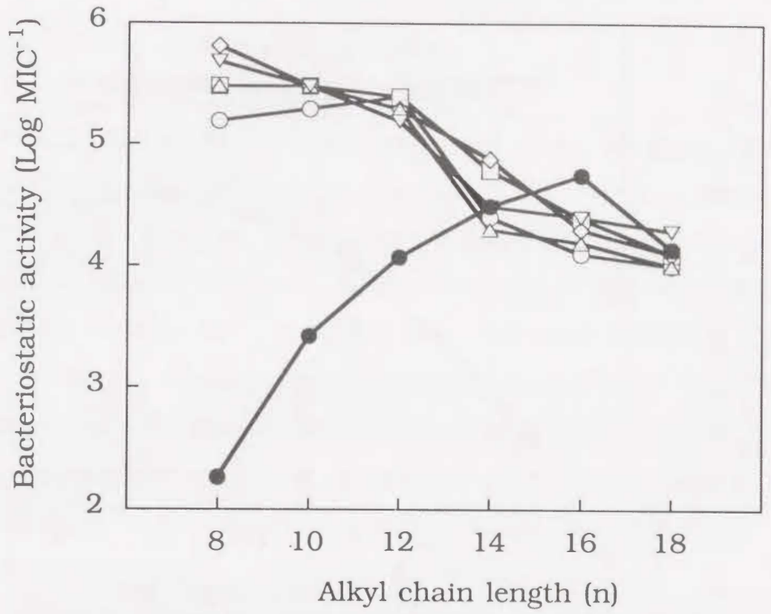


Fig. 5-2. Effect of alkyl chain length (n) on the bacteriostatic activity of 4DTBP-m,n and P-n against stationary-phase cells of *E. coli* K12 W3110. MICs were measured by a broth dilution method with nutrient broth at 37℃ for 24 h. ○, 4DTBP-3,n; □, 4DTBP-4,n; ◇, 4DTBP-6,n; △, 4DTBP-8,n; ▽, 4DTBP-10,n; ●, P-n.

QACs, which have two hydrophobic alkyl chains, this could explain why the activities increased with shorter alkyl chain length. The bis-QAC that showed the strongest activity was 4DTBP-6,8 (MIC = 1.6 μM). It is possible that a hydrophobic association between 4DTBP-m,n (n = 14-18) and medium components caused a decrease in the effective concentration of these compounds in the measurement system.

Further, the effect of alkyl chain length of 4DTBP-m,n and P-n on the activity was investigated in terms of MBC in the absence of medium components. The bactericidal activities, as well as the bacteriostatic activities, of P-n were remarkably influenced by the

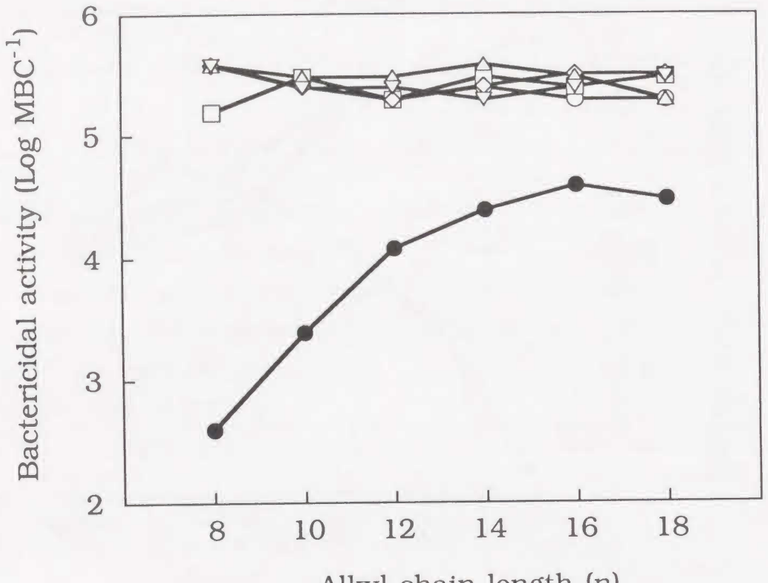


Fig. 5-3. Effect of alkyl chain length (n) on the bactericidal activity of 4DTBP-m,n and P-n against exponential-phase cells of *E. coli* K12 W3110. MBCs were measured by a dilution method at 30℃ for 30 min. ○, 4DTBP-3,n; □, 4DTBP-4,n; ◇, 4DTBP-6,n; △, 4DTBP-8,n; ▽, 4DTBP-10,n; ●, P-n.

alkyl chain length, as shown in Fig. 5-3. The activities of 4DTBP-m,n, however, were not influenced by the alkyl group or the methylene group length. This result supports the idea that the lowering of the bacteriostatic activity of the tested bis-QACs was caused by hydrophobic interaction with the medium components. Though the bactericidal activity of P-18 was lower than that of P-16, the difference was small, compared with that in the bacteriostatic activities (Fig. 5-2). 4DTBP-6,8 (MBC = 2.6 μM) was again the most potent of all and the activity was about 10 times that of P-16.

5-3-4. *Effect of pH on bactericidal activity*

The MBCs of 4DTBP-6,12 and P-12 were measured using 0.05 M phosphate buffer (pH: 5, 6, 7, 8 and 8.5). As shown in Fig. 5-4, 4DTBP-6,12 maintained high activity in the pH range from 5 to 8.5. On the other hand, the activity of P-12 was strongly affected by the pH of the test solution, and it was significantly enhanced with a rise of pH. It is well-known that QACs such as P-12 are more effective in alkaline solution than in acidic solution (Kourai et al., 1994c). This is one of the disadvantages of QACs. However, 4DTBP-6,12 showed a low dependence of the bactericidal activity on pH, possibly due to its dimeric structure.

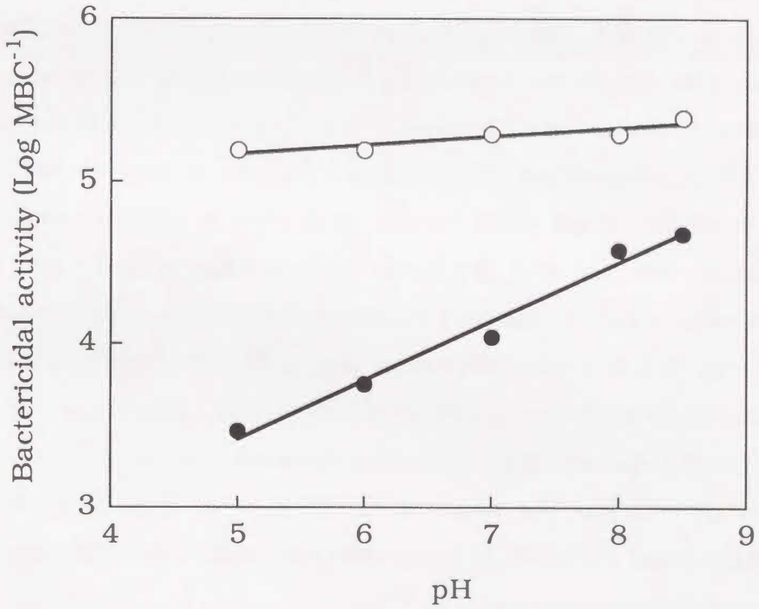


Fig. 5-4. Effect of pH on the bactericidal activity of 4DTBP-6,12 (○) and P-12 (●) against exponential-phase cells of *E. coli* K12 W3110. MBCs were measured at 30℃ for 30 min using 0.05 M phosphate buffer.

5-3-5. *Effect of temperature on bactericidal activity*

Generally, an increase in temperature tends to enhance the bactericidal activities of QACs, because the temperature is closely related to the fluidity of the bacterial cell membrane and most QACs interact with the membrane to exhibit bactericidal action. To investigate the interaction between the bacterial membrane and the bis-QACs synthesized in this study, the bactericidal activities of 4DTBP-6,12 and P-12 were measured at various temperatures. The following results should not have been influenced by bacterial growth since the MBCs were measured in a suspension without nutrient substances for 30 min. The plot of log MBC^{-1} against the reciprocal of the absolute temperature (T^{-1}) for P-12 gave two straight lines, as can be seen in Fig. 5-5. The temperature at the intersection of two lines seems to be the phase transition temperature of the bacterial cell membrane. On the other hand, the activity of 4DTBP-6,12 was not affected by temperature. This suggests that the activity of 4DTBP-6,12 is not dependent on the membrane fluidity. In addition, we have previously reported that the bactericidal activity of QACs can be regarded as a rate of the bactericidal action (Kourai et al., 1985a). Thus, Fig. 5-5 is a quasi Arrhenius plot and each slope shows the apparent activation energy of bactericidal action. Both slopes for P-12 are very large, which indicates that the activation energies of P-12 are large, whereas the slope of 4DTBP-6,12 is very small. It is concluded that 4DTBP-6,12 has a different bactericidal mechanism from the common QACs such as P-12.

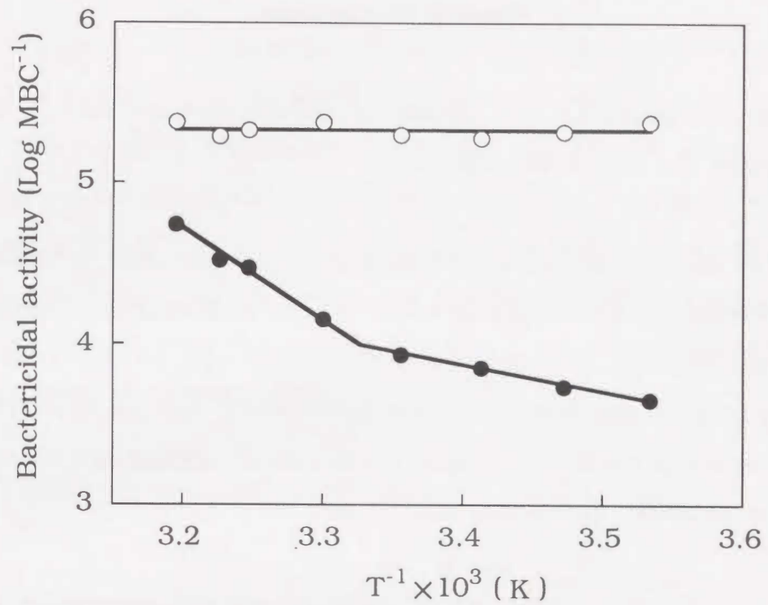


Fig. 5-5. Effect of temperature on the bactericidal activity of 4DTBP-6,12 (○) and P-12 (●) against exponential-phase cells of *E. coli* K12 W3110.

General Conclusions

Several important and interesting results were obtained in this study on the antimicrobial QACs:

1. The bactericidal activity of *N*-dodecylpyridinium iodide derivatives synthesized in this study was enhanced by introducing the electron-releasing groups, such as amino and methyl groups, into the pyridine ring. Also, it was found from the QSAR study that the derivatives having a high electron density on the quaternary ammonium nitrogen atom, exhibited a high antibacterial activity.

2. The turbidity increment in the bacterial cell suspension was induced by the addition of QACs. The ability of QACs was defined as bacterioclastic activity in this study. It was considered that the bacterioclastic action is one stage of the bactericidal action of QACs.

3. The activity of a homologous series of QAC was dependent on the molecular hydrophobicity. Similarly, the activity had a linear dependence to the hydrophobicity of bacterial cell surfaces. That is, QACs were more effective against the bacteria having hydrophobic surfaces than those having hydrophilic surfaces.

4. The influence of the pH value of the test condition on the activity of QACs is reduced by the introduction of relatively large substituents, such as benzylamino group, into the pyridine ring.

5. Bis-QACs, 4,4'-(α,ω-polymethylenedithio)bis(1-alkylpyridinium iodide)s (4DTBP-m,n), having two pyridinium moieties and two hydrophobic alkyl chains were synthesized. 4DTBP-m,n showed a potent and broad antimicrobial activity and spectra, compared with the mono-QACs. Further, the activity was not influenced by their molecular hydrophobicity or the test conditions (temperature and pH). It was assumed that 4DTBP-m,n has a different bactericidal action from the common QACs.

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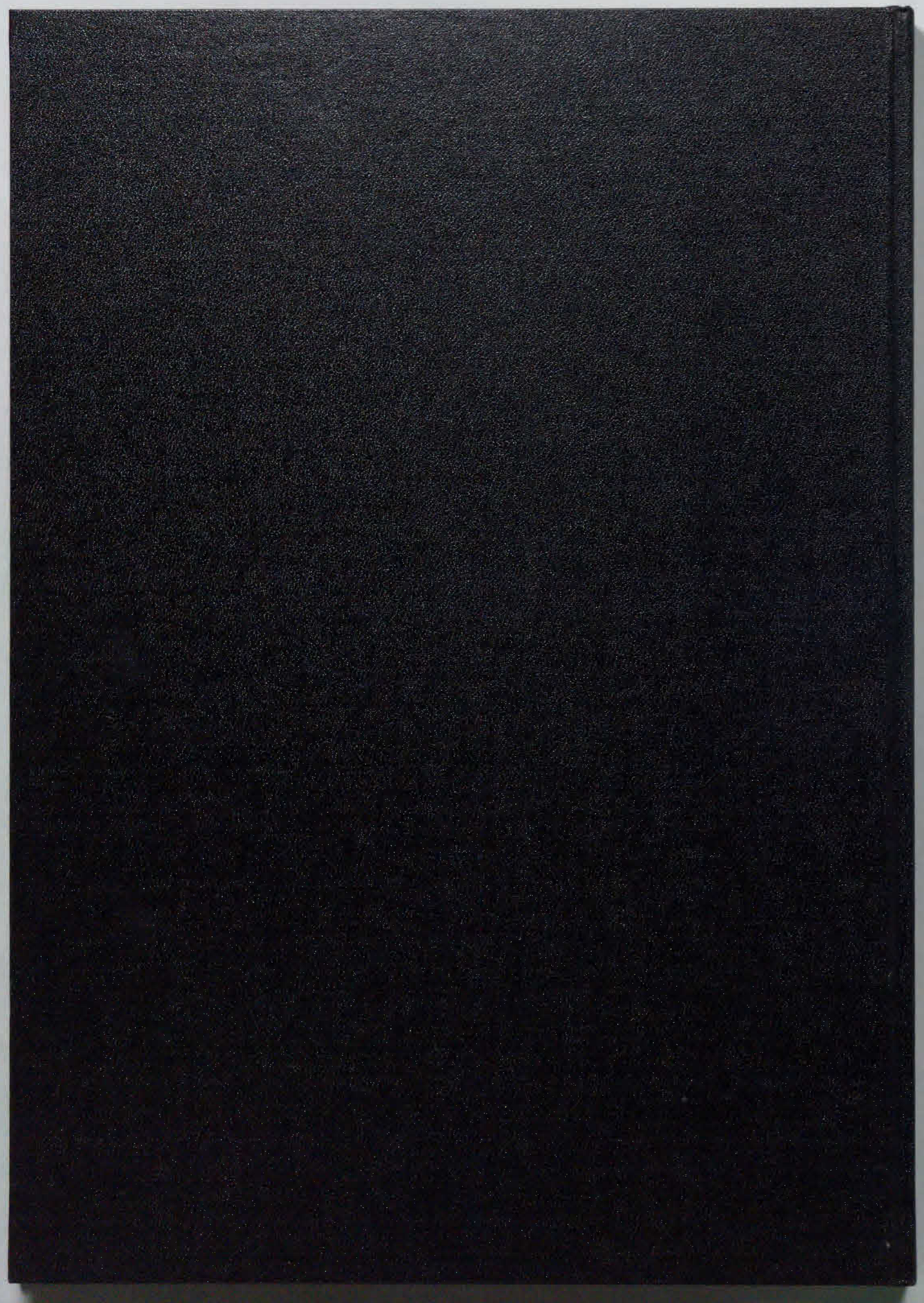

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様式9

論 文 審 査 の 結 果 の 要 旨

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学位論文題目

Studies on Antimicrobial Quaternary Ammonium Compounds

審査結果の要旨

この博士論文は抗微生物活性を有する第四アンモニウム塩（QAC）の合成とその作用特性に関するもので，QSAR を用いたドラッグデザインにより，非常に高い抗微生物活性と広い活性スペクトルを有する薬剤の開発および殺菌機構の解明を目的としたものである。

リード化合物として *N*-アルキルピリジニウム塩を用い，*N*-アルキル基をドデシル基に固定し，ピリジン核に様々な置換基を導入した薬剤を合成し，QSAR の手法により，優れた抗微生物活性を有する新規 QAC を見出している。特に殺菌活性が，置換基の種類と導入ポジションに大きく影響を受け，アミノ基やメチル基のような電子供与性基が 2 位または 4 位に導入されることによって活性が高められることを明らかにしている。さらにその殺菌活性は，ピリジンの酸解離定数（pKa）と，ピリジニウム窒素隣接メチレンプロトンのケミカルシフト値（δ ppm）との間に高い相関が存在することも見出している。また，これら薬剤が *Escherichia coli* K12 W3110 の細胞膜を短時間で破壊することを見出し，これを細胞破壊活性と定義して殺菌機構を定量的に解析し，細胞破壊現象が QAC の殺菌メカニズムの重要な 1 段階であることを見出している。

これらの知見をもとにピリジン核の 2 位または 4 位にベンジルアミノ基，アリルチオ基、あるいはアルキルチオ基を導入し，*N*-置換アルキル基の炭素数を変化させてそれらの抗微生物活性を測定し，リード化合物の *N*-アルキルピリジニウム塩よりも高い活性を見出している。さらに，ベンジルアミノ基のような大きな置換基の導入によって，幅広い pH 領域において高い殺菌活性を示す効果も見出している。

以上の，mono-QAC に関する研究結果をもとに， 2 個のアンモニウム窒素と 2 個のアルキル基を有する bis-QAC 型薬剤の合成法の確立とその抗微生物活性に関する研究を進め， 2 個のアルキルピリジニウム塩を 4 位でイオウを介して炭素数 3～10 のメチレン鎖で連結した新規 bis-QAC の合成に成功している。この bis-QAC はグラム陰性細菌，陽性細菌のいずれにも非常に高い殺菌活性と通常の抗カビ剤の TBZ よりも高い活性を示すことを明らかにした。さらに bis-QAC は pH，温度の影響を受けない高い殺菌活性をも見出し，医療現場での消毒剤として非常に有効であることを示した。さらに，この新規 bis-QAC は，リード化合物とは異なった殺菌機構を有することも明らかにした。

以上の本論文は博士（工学）の学位授与に値するものと判定する。